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# UTILITY PATENT APPLICATION TRANSMITTAL

(Only for new nonprovisional applications under 37 C.F.R. § 1.53(b))

Attorney Docket No.	10447-011
First Inventor or Application Identifier	THOR BORGFOR
Title	RICIN-LIKE TOXIN VARIANTS FOR TREATMENT OF ...
Express Mail Label No.	

## APPLICATION ELEMENTS

See MPEP chapter 600 concerning utility patent application contents.

1. ☒ \* Fee Transmittal Form (e.g., PTO/SB/17)  
(Submit an original and a duplicate for fee processing)
2. ☒ Specification [Total Pages 88]  
(preferred arrangement set forth below)
  - Descriptive title of the Invention
  - Cross References to Related Applications
  - Statement Regarding Fed sponsored R & D
  - Reference to Micro fiche Appendix
  - Background of the Invention
  - Brief Summary of the Invention
  - Brief Description of the Drawings (if filed)
  - Detailed Description
  - Claim(s)
  - Abstract of the Disclosure
3. ☒ Drawing(s) (35 U.S.C. 113) [Total Sheets 254]
4. Oath or Declaration [Total Pages 3]
  - a. ☒ Newly executed (original or copy)
  - b. ☐ Copy from a prior application (37 C.F.R. § 1.63(d))  
(for continuation/divisional with Box 16 completed)
    - i. ☐ DELETION OF INVENTOR(S)  
Signed statement attached deleting inventor(s) named in the prior application, see 37 C.F.R. §§ 1.63(d)(2) and 1.33(b).

\* NOTE FOR ITEMS 1 & 13: IN ORDER TO BE ENTITLED TO PAY SMALL ENTITY FEES, A SMALL ENTITY STATEMENT IS REQUIRED (37 C.F.R. § 1.27), EXCEPT IF ONE FILED IN A PRIOR APPLICATION IS RELIED UPON (37 C.F.R. § 1.28).

ADDRESS TO: Assistant Commissioner for Patents  
Box Patent Application  
Washington, DC 20231

5. ☐ Microfiche Computer Program (Appendix)
6. Nucleotide and/or Amino Acid Sequence Submission  
(if applicable, all necessary)
  - a. ☐ Computer Readable Copy
  - b. ☐ Paper Copy (identical to computer copy)
  - c. ☐ Statement verifying identity of above copies

## ACCOMPANYING APPLICATION PARTS

7. ☐ Assignment Papers (cover sheet & document(s))
8. ☐ 37 C.F.R. § 3.73(b) Statement of Power of Attorney  
(when there is an assignee)
9. ☐ English Translation Document (if applicable)
10. ☐ Information Disclosure Statement (IDS)/PTO-1449 [Copies of IDS Citations]
11. ☐ Preliminary Amendment
12. ☐ Return Receipt Postcard (MPEP 503)  
(Should be specifically itemized)
13. ☒ \* Small Entity Statement filed in prior application, Status still proper and desired  
(PTO/SB/09-12)
14. ☐ Certified Copy of Priority Document(s)  
(if foreign priority is claimed)
15. ☐ Other:

16. If a CONTINUING APPLICATION, check appropriate box, and supply the requisite information below and in a preliminary amendment:

☐ Continuation ☐ Divisional ☒ Continuation-in-part (CIP) of prior application No: 09/403,752  
Prior application information: Examiner Group / Art Unit:

For CONTINUATION or DIVISIONAL APPS only: The entire disclosure of the prior application, from which an oath or declaration is supplied under Box 4b, is considered a part of the disclosure of the accompanying continuation or divisional application and is hereby incorporated by reference. The incorporation can only be relied upon when a portion has been inadvertently omitted from the submitted application parts.

## 17. CORRESPONDENCE ADDRESS

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Name (Print/Type)	John R. Rudolph	Registration No. (Attorney/Agent)	38.003
Signature		Date	April 13, 2000

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Our File No: 10447-011

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April 13, 2000

The Commissioner of Patents & Trademarks  
Washington, D.C., U.S.A. 20231

Dear Sir:

Transmitted herewith for filing is the patent application of

Inventor: **Thor Borgford**

For: **RICIN-LIKE TOXIN VARIANTS FOR TREATMENT OF CANCER, VIRAL OR PARASITIC INFECTIONS**

The following papers are also enclosed:

[X] 254 sheets of X Formal drawings \_\_\_\_\_ Informal drawings

[X] A signed declaration.

	Number Extra	Full Rate	Small Entity Rate	Total
Total Claims	39 - 20 = 19 X	\$ 18.00	\$ 9.00	\$171
Independent Claims	5 - 3 = 2 X	\$ 78.00	\$ 39.00	\$78
Multiple Claims	Flat Rate	\$260.00	\$130.00	\$0

Basic Fee = \$345.00  
TOTAL FEES = \$594.00

[X] Two Verified Statements Claiming Small Entity Status.

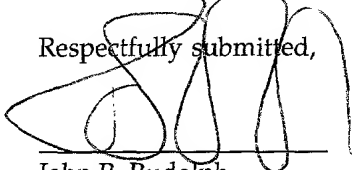
[X] The amount of \$594.00 is enclosed in our cheque No. 2860.

[X] The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Account No. 02-2095. This letter is enclosed in duplicate.

[X] Related application. This application is a \_\_\_\_\_ Division \_\_\_\_\_ Continuation  
X Continuation-in-part of application Serial No. 09/403,752, filed October 29, 1999.

[ ] Convention priority is requested based on U.S. Application Serial No. \_\_\_\_\_ filed \_\_\_\_\_, under 35 USC §119(e).

Respectfully submitted,

  
John R. Rudolph  
Registration No. 38,003

Applicant or Patentee: Thor Borgford  
 Serial or Patent No.: \_\_\_\_\_  
 Filed or Issued: \_\_\_\_\_  
 For: Ricin-Like Toxin Variants For Treatment of Cancer, Viral or Parasitic Infections

**VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS**  
**(37 CFR 1.9(f) and 1.27(b)) - INDEPENDENT INVENTOR**

As a below named inventor, I hereby declare that I qualify as an independent inventor as defined in 37 CFR 1.9(c) for purposes of paying reduced fees under section 41(a) and (b) of Title 35, United States Code, to the Patent and Trademark Office with regard to the invention entitled Ricin-Like Toxin Variants For Treatment of Cancer, Viral or Parasitic Infections described in

- ☒ the specification filed herewith  
☐ Application serial no. \_\_\_\_\_, filed \_\_\_\_\_  
☐ patent no. \_\_\_\_\_, issued \_\_\_\_\_

I have not assigned, granted, conveyed or licensed and am under no obligation under contract or law to assign, grant, convey or license, any rights in the invention to any person who could not be classified as an independent inventor under 37 CFR 1.9(c) if that person had made the invention, or to any concern which would not qualify as a small business concern under 37 CFR 1.9(d) or a nonprofit organization under 37 CFR 1.9(e).

Each person, concern or organization to which I have assigned, granted, conveyed, or licensed or am under an obligation under contract or law to assign, grant, convey, or license any rights in the invention is listed below:

- ☐ no such person, concern, or organization  
☒ persons, concerns or organizations listed below\*

\*NOTE: Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities. (37 CFR 1.27)

FULL NAME De Novo Enzyme Corporation  
 ADDRESS 8081 Lougheed Highway, Burnaby, B.C. V5A 1W9, Canada  
☐ INDIVIDUAL ☒ SMALL BUSINESS CONCERN ☐ NONPROFIT ORGANIZATION

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28(b))

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

Thor Borgford  
 Name of Inventor

X Thor Borgford  
 Signature of Inventor

X APRIL 13, 2000  
 Date

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Applicant or Patentee: Thor Borgford

Serial or Patent No.: \_\_\_\_\_

Filed or Issued: \_\_\_\_\_

For: Ricin-Like Toxin Variants For Treatment of Cancer, Viral or Parasitic Infections

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS  
(37 CFR 1.9(f) and 1.27 (a)) - SMALL BUSINESS CONCERN

I hereby declare that I am

☐ the owner of the small business concern identified below:

☒ an official of the small business concern empowered to act on behalf of the concern identified below:

NAME OF CONCERN De Novo Enzyme Corporation

ADDRESS OF CONCERN 8081 Lougheed Highway, Burnaby, B.C., V5A 1W9, Canada

I hereby declare that the above identified small business concern qualifies as a small business concern as defined in 13 CFR 121.3-18, and reproduced in 37 CFR 1.9(d), for purposes of paying reduced fees under section 41(a) and (b) of Title 35, United States Code, in that the number of employees of the concern, including those of its affiliates, does not exceed 300 persons. For purposes of this statement, (1) the number of employees of the business concern is the average over the previous fiscal year of the concern of the persons employed on a full-time, part-time or temporary basis during each of the pay periods of the fiscal year, and (2) concerns are affiliates of each other when either, directly or indirectly, one concern controls or has the power to control the other, or a third party or parties controls or has the power to control both.

I hereby declare that rights under contract or law have been conveyed to and remain with the small business concern identified above with regard to the invention, entitled Ricin-Like Toxin Variants For Treatment of Cancer, Viral or Parasitic Infections by inventor Thor Borgford described in

☒ the specification filed herewith

☐ PCT application serial no. \_\_\_\_\_ filed \_\_\_\_\_

☐ patent no. \_\_\_\_\_ issued \_\_\_\_\_

If the rights held by the above identified small business concern are not exclusive, each individual, concern or organization having rights to the invention is listed below and no rights to the invention are held by any person, other than the inventor, who could not qualify as a small business concern under 37 CFR 1.9(c) or by any concern which would not qualify as a small business concern under 37 CFR 1.9(d) or a nonprofit organization under 37 CFR 1.9(e). NOTE: Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities. (37 CFR 1.27)

NAME \_\_\_\_\_

ADDRESS \_\_\_\_\_

☐ INDIVIDUAL ☐ SMALL BUSINESS CONCERN ☐ NONPROFIT ORGANIZATION

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28 (b))

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

NAME OF PERSON SIGNING CURTIS BRAUN

TITLE OF PERSON OTHER THAN OWNER DIRECTOR OF OPERATIONS

ADDRESS OF PERSON SIGNING 11519 140 A STREET SURREY BC CANADA V2R 1J1

SIGNATURE [Signature]

DATE APRIL 13, 2000

00447-011-10447-011



B&P File No. 10447-011

**BERESKIN & PARR**

**UNITED STATES**

**Title: RICIN-LIKE TOXIN VARIANTS FOR TREATMENT OF  
CANCER, VIRAL OR PARASITIC INFECTIONS**

**Inventor: THOR BORGFORD**

004470-157550

**Title: RICIN-LIKE TOXIN VARIANTS FOR TREATMENT OF  
CANCER, VIRAL OR PARASITIC INFECTIONS**

This is a continuation-in-part of U.S. patent application serial  
No. 09/403,752 filed October 29, 1999.

5 **FIELD OF THE INVENTION**

The invention relates to proteins useful as therapeutics  
against cancer, viral infections, parasitic and fungal infections. The proteins  
contain A and B chains of a ricin-like toxin linked by a linker sequence that  
is specifically cleaved and activated by proteases specific to disease-  
10 associated pathogens or cells.

**BACKGROUND OF THE INVENTION**

Bacteria and plants are known to produce cytotoxic proteins  
which may consist of one, two or several polypeptides or subunits. Those  
proteins having a single subunit may be loosely classified as Type I  
15 proteins. Many of the cytotoxins which have evolved two subunit  
structures are referred to as type II proteins (Saelinger, C.B. in Trafficking  
of Bacterial Toxins (eds. Saelinger, C.B.) 1-13 (CRC Press Inc., Boca Raton,  
Florida, 1990). One subunit, the A chain, possesses the toxic activity  
whereas the second subunit, the B chain, binds cell surfaces and mediates  
20 entry of the toxin into a target cell. A subset of these toxins kill target cells  
by inhibiting protein biosynthesis. For example, bacterial toxins such as  
diphtheria toxin or Pseudomonas exotoxin inhibit protein synthesis by  
inactivating elongation factor 2. Plant toxins such as ricin, abrin, and  
bacterial toxin Shiga toxin, inhibit protein synthesis by directly inactivating  
25 the ribosomes (Olsnes, S. & Phil, A. in Molecular action of toxins and  
viruses (eds. Cohen, P. & vanHeyningen, S.) 51-105 Elsevier Biomedical  
Press, Amsterdam, 1982).

Ricin, derived from the seeds of *Ricinus communis* (castor oil  
plant), may be the most potent of the plant toxins. It is estimated that a  
30 single ricin A chain is able to inactivate ribosomes at a rate of 1500  
ribosomes/minute. Consequently, a single molecule of ricin is enough to  
kill a cell (Olsnes, S. & Phil, A. in Molecular action of toxins and viruses  
(eds. Cohen, P. & vanHeyningen, S.) (Elsevier Biomedical Press,  
Amsterdam, 1982). The ricin toxin is a glycosylated heterodimer consisting

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of A and B chains with molecular masses of 30,625 Da and 31,431 Da linked by a disulphide bond. The A chain of ricin has an N-glycosidase activity and catalyzes the excision of a specific adenine residue from the 28S rRNA of eukaryotic ribosomes (Endo, Y. & Tsurugi, K. J., *Biol. Chem.* 262:8128  
5 (1987)). The B chain of ricin, although not toxic in itself, promotes the toxicity of the A chain by binding to galactose residues on the surface of eukaryotic cells and stimulating receptor-mediated endocytosis of the toxin molecule (Simmons et al., *Biol. Chem.* 261:7912 (1986)). Once the toxin molecule consisting of the A and B chains is internalized into the cell via  
10 clathrin-dependent or independent mechanisms, the greater reduction potential within the cell induces a release of the active A chain, eliciting its inhibitory effect on protein synthesis and its cytotoxicity (Emmanuel, F. et al., *Anal. Biochem.* 173: 134-141 (1988); Blum, J.S. et al., *J. Biol. Chem.* 266: 22091-22095 (1991); Fiani, M.L. et al., *Arch. Biochem. Biophys.* 307: 225-230  
15 (1993)). Empirical evidence suggests that activated toxin (e.g. ricin, shiga toxin and others) in the endosomes is transcytosed through the trans-Golgi network to the endoplasmic reticulum by retrograde transport before the A chain is translocated into the cytoplasm to elicit its action (Sandvig, K. & van Deurs, B., *FEBS Lett.* 346: 99-102 (1994)).  
20 Protein toxins are initially produced in an inactive, precursor form. Ricin is initially produced as a single polypeptide (preproricin) with a 35 amino acid N-terminal presequence and 12 amino acid linker between the A and B chains. The pre-sequence is removed during translocation of the ricin precursor into the endoplasmic reticulum (Lord, J.M., *Eur. J. Biochem.* 146:403-409 (1985) and Lord, J.M., *Eur. J. Biochem.* 146:411-416  
25 (1985)). The proricin is then translocated into specialized organelles called protein bodies where a plant protease cleaves the protein at a linker region between the A and B chains (Lord, J.M. et al., *FASAB Journal* 8:201-208 (1994)). The two chains, however, remain covalently attached by an  
30 interchain disulfide bond (cysteine 259 in the A chain to cysteine 4 in the B chain) and mature disulfide linked ricin is stored in protein bodies inside the plant cells. The A chain is inactive in proricin (O'Hare, M. et al., *FEBS Lett.* 273:200-204 (1990)) and it is inactive in the disulfide-linked mature ricin (Richardson, P.T. et al., *FEBS Lett.* 255:15-20 (1989)). The ribosomes of the

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castor bean plant are themselves susceptible to inactivation by ricin A chain; however, as there is no cell surface galactose to permit B chain recognition the A chain cannot re-enter the cell. The exact mechanism of A chain release and activation in target cell cytoplasm is not known (Lord, J.M. et al., *FASAB Journal* 8:201-208 (1994)). However, it is known that for activation to take place the disulfide bond between the A and B chains must be reduced and, hence, the linkage between subunits broken.

Diphtheria toxin is produced by *Corynebacterium diphtheriae* as a 535 amino acid polypeptide with a molecular weight of approximately 58kD (Greenfield, L. et al., *Proc. Natl. Acad. Sci. USA* 80:6853-6857 (1983); Pastan, I. et al., *Annu. Rev. Biochem.* 61:331-354 (1992); Collier, R.J. & Kandel, J., *J. Biol. Chem.* 246:1496-1503 (1971)). It is secreted as a single-chain polypeptide consisting of 2 functional domains. Similar to proricin, the N-terminal domain (A-chain) contains the cytotoxic moiety whereas the C-terminal domain (B-chain) is responsible for binding to the cells and facilitates toxin endocytosis. Conversely, the mechanism of cytotoxicity for diphtheria toxin is based on ADP-ribosylation of EF-2 thereby blocking protein synthesis and producing cell death. The 2 functional domains in diphtheria toxin are linked by an arginine-rich peptide sequence as well as a disulphide bond. Once the diphtheria toxin is internalized into the cell, the arginine-rich peptide linker is cleaved by trypsin-like enzymes and the disulphide bond (Cys 186-201) is reduced. The cytotoxic domain is subsequently translocated into the cytosol substantially as described above for ricin and elicits ribosomal inhibition and cytotoxicity.

*Pseudomonas* exotoxin is also a 66kD single-chain toxin protein secreted by *Pseudomonas aeruginosa* with a similar mechanism of cytotoxicity to that of diphtheria toxin (Pastan, I. et al., *Annu. Rev. Biochem.* 61:331-354 (1992); Ogata, M. et al., *J. Biol. Chem.* 267:25396-25401 (1992); Vagil, M.L. et al., *Infect. Immunol.* 16:353-361 (1977)). *Pseudomonas* exotoxin consists of 3 conjoint functional domains. The first domain Ia (amino acids 1-252) is responsible for cell binding and toxin endocytosis, a second domain II (amino acids 253-364) is responsible for toxin translocation from the endocytic vesicle to the cytosol, and a third domain III (amino acids 400-613) is responsible for protein synthesis inhibition and cytotoxicity.

After *Pseudomonas* exotoxin enters the cell, the liberation of the cytotoxic domain is effected by both proteolytic cleavage of a polypeptide sequence in the second domain (near Arg 279) and the reduction of the disulphide bond (Cys 265-287) in the endocytic vesicles. In essence, the overall pathway to cytotoxicity is analogous to diphtheria toxin with the exception that the toxin translocation domain in *Pseudomonas* exotoxin is structurally distinct.

Class 2 ribosomal inhibitory proteins (RIP-2) constitute other toxins possessing distinct functional domains for cytotoxicity and cell binding/toxin translocation which include abrin, modeccin, volkensin, (Sandvig, K. et al., *Biochem. Soc. Trans.* 21:707-711 (1993)) and mistle toe lectin (viscumin) (Olsnes, S. & Phil, A. in *Molecular action of toxins and viruses* (eds. Cohen, P. & vanHeyningen, S.) 51-105 Elsevier Biomedical Press, Amsterdam, 1982; and Fodstad, et al. *Canc. Res.* 44:862 (1984)). Some toxins such as Shiga toxin and cholera toxin also have multiple polypeptide chains responsible for receptor binding and endocytosis.

The ricin gene has been cloned and sequenced, and the X-ray crystal structures of the A and B chains have been described (Rutenber, E. et al. *Proteins* 10:240-250 (1991); Weston et al., *Mol. Bio.* 244:410-422, 1994; Lamb and Lord, *Eur. J. Biochem.* 14:265 (1985); Halling, K. et al. *Nucleic Acids Res.* 13:8019 (1985)). Similarly, the genes for diphtheria toxin and *Pseudomonas* exotoxin have been cloned and sequenced, and the 3-dimensional structures of the toxin proteins have been elucidated and described (Columblatti, M. et al., *J. Biol. Chem.* 261:3030-3035 (1986); Allured, V.S. et al., *Proc. Natl. Acad. Sci. USA* 83:1320-1324 (1986); Gray, G.L. et al., *Proc. Natl. Acad. Sci. USA* 81:2645-2649 (1984); Greenfield, L. et al., *Proc. Natl. Acad. Sci. USA* 80:6853-6857 (1983); Collier, R.J. et al., *J. Biol. Chem.* 257:5283-5285 (1982)).

The potential of bacterial and plant toxins for inhibiting mammalian retroviruses, particularly acquired immunodeficiency syndrome (AIDS), has been investigated. Bacterial toxins such as *Pseudomonas* exotoxin-A and subunit A of diphtheria toxin; dual chain ribosomal inhibitory plant toxins such as ricin, and single chain ribosomal inhibitory proteins such as trichosanthin and pokeweed antiviral protein

have been used for the elimination of HIV infected cells (Olson et al., *AIDS Res. and Human Retroviruses* 7:1025-1030 (1991)). The high toxicity of these toxins for mammalian cells, combined with a lack of specificity of action poses a major problem to the development of pharmaceuticals incorporating the toxins, such as immunotoxins.

Due to their extreme toxicity there has been much interest in making ricin-based immunotoxins as therapeutic agents for specifically destroying or inhibiting infected or tumourous cells or tissues (Vitetta et al., *Science* 238:1098-1104(1987)). An immunotoxin is a conjugate of a specific cell binding component, such as a monoclonal antibody or growth factor and the toxin in which the two protein components are covalently linked. Generally, the components are chemically coupled. However, the linkage may also be a peptide or disulfide bond. The antibody directs the toxin to cell types presenting a specific antigen thereby providing a specificity of action not possible with the natural toxin. Immunotoxins have been made both with the entire ricin molecule (i.e. both chains) and with the ricin A chain alone (Spooner et al., *Mol. Immunol.* 31:117-125, (1994)).

Immunotoxins made with the ricin dimer (IT-Rs) are more potent toxins than those made with only the A chain (IT-As). The increased toxicity of IT-Rs is thought to be attributed to the dual role of the B chains in binding to the cell surface and in translocating the A chain to the cytosolic compartment of the target cell (Vitetta et al., *Science* 238:1098-1104 (1987); Vitetta & Thorpe, *Seminars in Cell Biology* 2:47-58 (1991)). However, the presence of the B chain in these conjugates also promotes the entry of the immunotoxin into nontarget cells. Even small amounts of B chain may override the specificity of the cell-binding component as the B chain will bind nonspecifically to galactose associated with N-linked carbohydrates, which is present on most cells. IT-As are more specific and safer to use than IT-Rs. However, in the absence of the B chain the A chain has greatly reduced toxicity. Due to the reduced potency of IT-As as compared to IT-Rs, large doses of IT-As must be administered to patients. The large doses frequently cause immune responses and production of neutralizing antibodies in patients (Vitetta et al., *Science* 238:1098-1104

(1987)). IT-As and IT-Rs both suffer from reduced toxicity as the A chain is not released from the conjugate into the target cell cytoplasm.

A number of immunotoxins have been designed to recognize antigens on the surfaces of tumour cells and cells of the immune system (Pastan et al., *Annals New York Academy of Sciences* 758:345-353 (1995)). A major problem with the use of such immunotoxins is that the antibody component is its only targeting mechanism and the target antigen is often found on non-target cells (Vitetta et al., *Immunology Today* 14:252-259 (1993)). Also, the preparation of a suitable specific cell binding component may be problematic. For example, antigens specific for the target cell may not be available and many potential target cells and infective organisms can alter their antigenic make up rapidly to avoid immune recognition. In view of the extreme toxicity of proteins such as ricin, the lack of specificity of the immunotoxins may severely limit their usefulness as therapeutics for the treatment of cancer and infectious diseases.

The insertion of intramolecular protease cleavage sites between the cytotoxic and cell-binding components of a toxin can mimic the way that the natural toxin is activated. European patent application no. 466,222 describes the use of maize-derived pro-proteins which can be converted into active form by cleavage with extracellular blood enzymes such as factor Xa, thrombin or collagenase. Garred, O. et al. (*J. Biol. Chem.* 270:10817-10821 (1995)) documented the use of a ubiquitous calcium-dependent serine protease, furin, to activate shiga toxin by cleavage of the trypsin-sensitive linkage between the cytotoxic A-chain and the pentamer of cell-binding B-units. Westby et al. (*Bioconjugate Chem.* 3:375-381 (1992)) documented fusion proteins which have a specific cell binding component and proricin with a protease sensitive cleavage site specific for factor Xa within the linker sequence. O'Hare et al. (*FEBS Lett.* 273:200-204 (1990)) also described a recombinant fusion protein of RTA and staphylococcal protein A joined by a trypsin-sensitive cleavage site. In view of the ubiquitous nature of the extracellular proteases utilized in these approaches, such artificial activation of the toxin precursor or immunotoxin does not confer a mechanism for intracellular toxin

activation and the problems of target specificity and adverse immunological reactions to the cell-binding component of the immunotoxin remain.

In a variation of the approach of insertion of intramolecular protease cleavage sites on proteins which combine a binding chain and a toxic chain, Leppla, S.H. et al. (Bacterial Protein Toxins zbl.bakt.suppl. 24:431-442 (1994)) suggest the replacement of the native cleavage site of the protective antigen (PA) produced by *Bacillus anthracis* with a cleavage site that is recognized by cells that contain a particular protease. PA, recognizes, binds, and thereby assists in the internalization of lethal factor (LF) and edema toxin (ET). also produced by *Bacillus anthracis*. However, this approach is wholly dependent on the availability of LF, or ET and PA all being localized to cells wherein the modified PA can be activated by the specific protease. It does not confer a mechanism for intracellular toxin activation and presents a problem of ensuring sufficient quantities of toxin for internalization in target cells.

The *in vitro* activation of a *Staphylococcus*-derived pore-forming toxin,  $\alpha$ -hemolysin by extracellular tumour-associated proteases has been documented (Panchel, R.G. et al., *Nature Biotechnology* 14:852-857 (1996)). Artificial activation of  $\alpha$ -hemolysin *in vitro* by said proteases was reported but the actual activity and utility of  $\alpha$ -hemolysin in the destruction of target cells were not demonstrated.

Hemolysin does not inhibit protein synthesis but is a heptameric transmembrane pore which acts as a channel to allow leakage of molecules up to 3 kD thereby disrupting the ionic balances of the living cell. The  $\alpha$ -hemolysin activation domain is likely located on the outside of the target cell (for activation by extracellular proteases). The triggering mechanism in the disclosed hemolysin precursor does not involve the intracellular proteolytic cleavage of 2 functionally distinct domains. Also, the proteases used for the  $\alpha$ -hemolysin activation are ubiquitously secreted extracellular proteases and toxin activation would not be confined to activation in the vicinity of diseased cells. Such widespread activation of the toxin does not confer target specificity and limits the usefulness of said  $\alpha$ -hemolysin toxin as therapeutics due to systemic toxicity.



A variety of proteases specifically associated with malignancy, viral infections and parasitic infections have been identified and described. For example, cathepsin is a family of serine, cysteine or aspartic endopeptidases and exopeptidases which has been implicated to  
5 play a primary role in cancer metastasis (Schwartz, M.K., *Clin. Chim. Acta* 237:67-78 (1995); Spiess, E. et al., *J. Histochem. Cytochem.* 42:917-929 (1994); Scarborough, P.E. et al., *Protein Sci.* 2:264-276 (1993); Sloane, B.F. et al., *Proc. Natl. Acad. Sci. USA* 83:2483-2487 (1986); Mikkelsen, T. et al., *J. Neurosurg* 83:285-290 (1995)). Matrix metalloproteinases (MMPs or matrixins) are  
10 zinc-dependent proteinases consisting of collagenases, matrilysin, stromelysins, gelatinases and macrophage elastase (Krane, S.M., *Ann. N.Y. Acad. Sci.* 732:1-10 (1994); Woessner, J.F., *Ann. N.Y. Acad. Sci.* 732:11-21 (1994); Carvalho, K. et al., *Biochem. Biophys. Res. Comm.* 191:172-179 (1993); Nakano, A. et al. *J. of Neurosurg*, 83:298-307 (1995); Peng, K-W, et al.  
15 *Human Gene Therapy*, 8:729-738 (1997); More, D.H. et al. *Gynaecologic Oncology*, 65:78-82 (1997)). These proteases are involved in pathological matrix remodeling. Under normal physiological conditions, regulation of matrixin activity is effected at the level of gene expression. Enzymatic activity is also controlled stringently by tissue inhibitors of  
20 metalloproteinases (TIMPs) (Murphy, G. et al., *Ann. N.Y. Acad. Sci.* 732:31-41 (1994)). The expression of MMP genes is reported to be activated in inflammatory disorders (e.g. rheumatoid arthritis) and malignancy.

In malaria, parasitic serine and aspartic proteases are involved in host erythrocyte invasion by the *Plasmodium* parasite and in  
25 hemoglobin catabolism by intraerythrocytic malaria (O'Dea, K.P. et al., *Mol. Biochem. Parasitol.* 72:111-119 (1995); Blackman, M.J. et al., *Mol. Biochem. Parasitol.* 62:103-114 (1993); Cooper, J.A. et al., *Mol. Biochem. Parasitol.* 56:151-160 (1992); Goldberg, D.E. et al., *J. Exp. Med.* 173:961-969 (1991)). *Schistosoma mansoni* is also a pathogenic parasite which causes  
30 schistosomiasis or bilharzia. Elastinolytic proteinases have been associated specifically with the virulence of this particular parasite (McKerrow, J.H. et al., *J. Biol. Chem.* 260:3703-3707 (1985)).

Welch, A.R. et al. (*Proc. Natl. Acad. Sci. USA* 88:10797-10800 (1991)) has described a series of viral proteases which are specifically

associated with human cytomegalovirus, human herpesviruses, Epstein-Barr virus, varicella zoster virus-I. and infectious laryngotracheitis virus. These proteases possess similar substrate specificity and play an integral role in viral scaffold protein restructuring in capsid assembly and virus maturation. Other viral proteases serving similar functions have also been documented for human T-cell leukemia virus (Blaha, I. et al., *FEBS Lett.* 309:389-393 (1992); Pettit, S.C. et al., *J. Biol. Chem.* 266:14539-14547 (1991)), hepatitis viruses (Hirowatari, Y. et al., *Anal. Biochem.* 225:113-120 (1995); Hirowatari, Y. et al., *Arch. Virol.* 133:349-356 (1993); Jewell, D.A. et al., *Biochemistry* 31:7862-7869 (1992)), poliomyelitis virus (Weidner, J.R. et al., *Arch. Biochem. Biophys.* 286:402-408 (1991)), and human rhinovirus (Long, A.C. et al., *FEBS Lett.* 258:75-78 (1989)).

*Candida* yeasts are dimorphic fungi which are responsible for a majority of opportunistic infections in AIDS patients (Holmberg, K. and Myer, R., *Scand. J. Infect. Dis.* 18:179-192 (1986)). Aspartic proteinases have been associated specifically with numerous virulent strains of *Candida* including *Candida albican*, *Candida tropicalis*, and *Candida parapsilosis* (Abad-Zapatero, C. et al., *Protein Sci.* 5:640-652 (1996); Cutfield, S.M. et al., *Biochemistry* 35:398-410 (1995); Ruchel, R. et al., *Zentralbl. Bakteriol. Mikrobiol. Hyg. I Abt. Orig. A.* 255:537-548 (1983); Remold, H. et al., *Biochim. Biophys. Acta* 167:399-406 (1968)), and the levels of these enzymes have been correlated with the lethality of the strain (Schreiber, B, et al., *Diagn. Microbiol. Infect. Dis.* 3:1-5 (1985)).

#### **SUMMARY OF THE INVENTION**

The invention relates to novel recombinant toxic proteins which are specifically toxic to diseased cells but do not depend for their specificity of action on a specific cell binding component. The recombinant proteins of the invention have an A chain of a ricin-like toxin linked to a B chain by a synthetic linker sequence which may be cleaved specifically by a protease localised in cells or tissues affected by a specific disease to liberate the toxic A chain thereby selectively inhibiting or destroying the diseased cells or tissues. The term diseased cells as used herein, includes cells affected by cancer, or infected by fungi, or viruses, including retroviruses, or parasites.

Toxin targeting using the recombinant toxic proteins of the invention takes advantage of the fact that many DNA viruses exploit host cellular transport mechanisms to escape immunological destruction. This is achieved by enhancing the retrograde translocation of host major histocompatibility complex (MHC) type I molecules from the endoplasmic reticulum into the cytoplasm (Bonifacino, J.S., *Nature* 384: 405-406 (1996); Wiertz, E.J. et al., *Nature* 384: 432-438 (1996)). The facilitation of retrograde transport in diseased cells by the virus can enhance the transcytosis and cytotoxicity of a recombinant toxic protein of the present invention thereby further reducing non-specific cytotoxicity and improving the overall safety of the product.

The recombinant toxic proteins of the present invention may be used to treat diseases including various forms of cancer such as T- and B-cell lymphoproliferative diseases, ovarian cancer, pancreatic cancer, head and neck cancer, squamous cell carcinoma, gastrointestinal cancer, breast cancer, prostate cancer, non small cell lung cancer, malaria, and diverse viral disease states associated with infection with human cytomegalovirus, hepatitis virus, herpes virus, human rhinovirus, infectious laryngotracheitis virus, poliomyelitis virus, or varicella zoster virus.

In one aspect, the present invention provides a purified and isolated nucleic acid having a nucleotide sequence encoding an A chain of a ricin-like toxin, a B chain of a ricin-like toxin and a heterologous linker amino acid sequence, linking the A and B chains. The linker sequence is not a native linker sequence of a ricin-like toxin, but rather a synthetic heterologous linker sequence containing a cleavage recognition site for a disease-specific protease. The A and or the B chain may be those of ricin.

In an embodiment, of the invention the cleavage recognition site is the cleavage recognition site for a cancer-associated protease. In particular embodiments, the linker amino acid sequence comprises SLLKSRMVPNFN or SLLIARRMPNFN cleaved by cathepsin B; SKLVQASASGVN or SSYLKASDAPDN cleaved by an Epstein-Barr virus protease; RPKPQQFFGLMN cleaved by MMP-3 (stromelysin); SLRPLALWRSFN cleaved by MMP-7 (matrilysin); SPQGIAGQRNFN

cleaved by MMP-9; DVDERDVGRGFASFL cleaved by a thermolysin-like  
MMP; SLPLGLWAPNFN cleaved by matrix metalloproteinase 2(MMP-2) ;  
SLLIFRSWANFN cleaved by cathepsin L; SGVVIATVIVIT cleaved by  
cathepsin D; SLGPQGIWGQFN cleaved by matrix metalloproteinase  
5 1(MMP-1); KKSPGRVVGGSV cleaved by urokinase-type plasminogen  
activator; PQGLLGAPGILG cleaved by membrane type 1  
matrixmetalloproteinase (MT-MMP);  
HGPEGLRVGFYESDVMGRGHARLVHVEEPHT cleaved by stromelysin 3  
(or MMP-11), thermolysin, fibroblast collagenase and stromelysin-1;  
10 GPQGLAGQRGIV cleaved by matrix metalloproteinase 13 (collagenase-3);  
GGSGQRGRKALE cleaved by tissue-type plasminogen activator(tPA);  
SLSALLSSDIFN cleaved by human prostate-specific antigen;  
SLPRFKIIGGFN cleaved by kallikrein (hK3); SLLGIAVPGNFN cleaved by  
neutrophil elastase; and FFKNIVTPRTPP cleaved by calpain (calcium  
15 activated neutral protease). The nucleic acid sequences for ricin A and B  
chains with each of the linker sequences are shown in Figures 2D, 35C, 3D,  
4D, 5D, 6D, 16D, 17D, 34C, 36C , 37C, 38C , 39C, 40C, 41C, 42C , 43C, 44C,  
45C, 46C and 47C, respectively.

In another embodiment, the cleavage recognition site is the  
20 cleavage recognition site for a protease associated with the malaria  
parasite, *Plasmodium falciparum*. In particular embodiments, the linker  
amino acid sequence comprises QVVQLQNYDEED; LPIFGESEDNDE;  
QVVTGEAISVTM; ALERTFLSFPTN or KFQDMLNISQHQ. The nucleic  
nucleotide sequences for ricin A and B chains with each of the linker  
25 sequences are shown in Figures 7D, 8D, 9D, 10D, and 11D.

In a another embodiment, the cleavage recognition site is  
the cleavage recognition site for a viral protease. The linker sequences  
preferably comprise the sequence Y-X-Y-A-Z wherein X is valine or  
leucine, Y is a polar amino acid, and Z is serine, asparagine or valine. In  
30 particular embodiments, the linker amino acid sequence comprises  
SGVVNASCLAN or SSYVKASVSPEN cleaved by a human  
cytomegalovirus protease; SALVNASSAHVN or STYLQASEKFKN cleaved  
by a herpes simplex 1 virus protease; SSILNASVPNFN cleaved by a  
human herpes virus 6 protease; SQDVNAVEASSN or SVYLQASTGYGN

cleaved by a varicella zoster virus protease; or SKYLQANEVITN cleaved by an infectious laryngotracheitis virus protease. The nucleic nucleotide sequences for ricin A and B chains with each of the linker sequences are shown in Figures 12D, 13D, 14D, 15D, 18D, 19D, 20D, and 22D.

5 In another embodiment, the cleavage recognition site is the cleavage recognition site for a hepatitis A viral protease. In particular embodiments, the linker amino acid sequence comprises SELRTQSFSNWN or SELWSQGIDDDN cleaved by a hepatitis A virus protease. The nucleic nucleotide sequences for ricin A and B chains with each of the linker  
10 sequences are shown in Figures 23D or 24D.

In another embodiment, the cleavage recognition site is the cleavage recognition site for a hepatitis C viral protease. In particular embodiments, the linker amino acid sequence comprises DLEVVTSTWVFN, DEMEECASHLFN, EDVVCCSMSYFN or  
15 KGWRL LAPITAY cleaved by a hepatitis C virus protease. The nucleic nucleotide sequences for ricin A and B chains with each of the linker sequences are shown in Figures 30C, 31C, 32C and 33C.

In another embodiment, the cleavage recognition site is the cleavage recognition site for a *Candida* fungal protease. In particular  
20 embodiments, the linker amino acid sequence is SKPAKFFRLNFN, SKPIEFFRLNFN or SKPAEFFALNFN cleaved by *Candida* aspartic protease. The nucleic nucleotide sequences for ricin A and B chains with the first linker sequence are shown in Figures 25D.

The present invention also provides a plasmid incorporating  
25 the nucleic acid of the invention. In an embodiment, the plasmid has the restriction map as shown in Figures 2A, 3A, 4A, 5A, 6A, 7A, 8A, 9A, 10A, 11A, 12A, 13A, 14A, 15A, 16A, 17A, 18A, 19A, 20A, 21A, 22A, 23A, 24A, or 25A.

In another embodiment, the present invention provides a  
30 baculovirus transfer vector incorporating the nucleic acid of the invention. In particular embodiments, the invention provides a baculovirus transfer vector having the DNA sequence as shown in Figure 1.

In a further embodiment, the present invention provides a baculovirus transfer vector incorporating the nucleic acid of the invention.

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In particular embodiments, the invention provides a baculovirus transfer vector having the restriction map as shown in Figures 2C, 3C, 4C, 5C, 6C, 7C, 8C, 9C, 10C, 11C, 12C, 13C, 14C, 15C, 16C, 17C, 18C, 19C, 20C, 21C, 22C, 23C, 24C, 25C, 30A, 31A, 32A, 33A, 34A, 35A, 36A, 37A, 38A, 39A, 40A, 41A, 42A, 43A, 44A, 45A, 46A, or 47A. or having the DNA sequence as shown in Figure 1.

In a further aspect, the present invention provides a recombinant protein comprising an A chain of a ricin-like toxin, a B chain of a ricin-like toxin and a heterologous linker amino acid sequence, linking the A and B chains, wherein the linker sequence contains a cleavage recognition site for a disease-specific protease (e.g., a cancer, viral, parasitic, or fungal protease). The A and/or the B chain may be those of ricin. In an embodiment, the cleavage recognition site is the cleavage recognition site for a cancer, viral or parasitic protease substantially as described above. In a particular embodiment, the cancer is T-cell or B-cell lymphoproliferative disease. In another particular embodiment, the virus is human cytomegalovirus, Epstein-Barr virus, hepatitis virus, herpes virus, human rhinovirus, infectious laryngotracheitis virus, poliomyelitis virus, or varicella zoster virus. In a further particular embodiment, the parasite is *Plasmodium falciparum*.

In a further aspect, the invention provides a pharmaceutical composition for treating a fungal infection, such as *Candida*, in a mammal comprising the recombinant protein of the invention and a pharmaceutically acceptable carrier, diluent or excipient.

In yet another aspect, the invention provides a method of inhibiting or destroying cells affected by a disease, which cells are associated with a disease specific protease, including cancer or infection with a virus, fungus, or a parasite each of which has a specific protease, comprising the steps of preparing a recombinant protein of the invention having a heterologous linker sequence which contains a cleavage recognition site for the disease-specific protease and administering the recombinant protein to the cells. In an embodiment, the cancer is T-cell or B-cell lymphoproliferative disease, ovarian cancer, pancreatic cancer, head and neck cancer, squamous cell carcinoma, gastrointestinal cancer, breast

cancer, prostate cancer, non small cell lung cancer. In another embodiment, the virus is human cytomegalovirus, Epstein-Barr virus, hepatitis virus, herpes virus, human rhinovirus, human T-cell leukemia virus, infectious laryngotracheitis virus, poliomyelitis virus, or varicella  
5 zoster virus. In another embodiment, the parasite is *Plasmodium falciparum*.

The present invention also relates to a method of treating a mammal with disease wherein cells affected by the disease are associated with a disease specific protease, including cancer or infection with a virus, fungus, or a parasite each of which has a specific protease by administering  
10 an effective amount of one or more recombinant proteins of the invention to said mammal.

Still further, a process is provided for preparing a pharmaceutical for treating a mammal with disease wherein cells affected by the disease are associated with a disease specific protease, including  
15 cancer or infection with a virus, fungus, or a parasite each of which has a specific protease comprising the steps of preparing a purified and isolated nucleic acid having a nucleotide sequence encoding an A chain of a ricin-like toxin, a B chain of a ricin-like toxin and a heterologous linker amino acid sequence, linking the A and B chains, wherein the linker  
20 sequence contains a cleavage recognition site for the disease-specific protease; introducing the nucleic acid into a host cell; expressing the nucleic acid in the host cell to obtain a recombinant protein comprising an A chain of a ricin-like toxin, a B chain of a ricin-like toxin and a heterologous linker amino acid sequence, linking the A and B chains wherein the linker  
25 sequence contains the cleavage recognition site for the disease-specific protease; and suspending the protein in a pharmaceutically acceptable carrier, diluent or excipient.

In an embodiment, a process is provided for preparing a pharmaceutical for treating a mammal with disease wherein cells affected  
30 by the disease are associated with a disease specific protease, including cancer or infection with a virus, fungus, or a parasite each of which has a specific protease comprising the steps of identifying a cleavage recognition site for the protease; preparing a recombinant protein comprising an A chain of a ricin-like toxin, a B chain of a ricin-like toxin and a heterologous

linker amino acid sequence, linking the A and B chains wherein the linker sequence contains the cleavage recognition site for the protease and suspending the protein in a pharmaceutically acceptable carrier, diluent or excipient.

5                   In a further aspect, the invention provides a pharmaceutical composition for treating for treating a mammal with disease wherein cells affected by the disease are associated with a disease specific protease, including cancer or infection with a virus, fungus, or a parasite comprising the recombinant protein of the invention and a pharmaceutically  
10 acceptable carrier, diluent or excipient.

Other features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples while indicating preferred embodiments of the invention are  
15 given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

#### **DESCRIPTION OF THE DRAWINGS**

The invention will be better understood with reference to  
20 the drawings in which:

Figure 1 shows the DNA sequence of the baculovirus transfer vector, pVL1393;

Figure 2A summarizes the cloning strategy used to generate the pAP-213 construct;

25                   Figure 2B shows the nucleotide sequence of the Cathepsin B linker regions of pAP-213;

Figure 2C shows the subcloning of the Cathepsin B linker variant into a baculovirus transfer vector;

30                   Figure 2D shows the DNA sequence of the pAP-214 insert containing ricin and the Cathepsin B linker;

Figure 3A summarizes the cloning strategy used to generate the pAP-215 construct;

Figure 3B shows the nucleotide sequence of the MMP-3 linker regions of pAP-215;

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Figure 3C shows the subcloning of the MMP-3 linker variant into a baculovirus transfer vector;

Figure 3D shows the DNA sequence of the pAP-216 insert containing ricin and the MMP-3 linker;

5           Figure 4A summarizes the cloning strategy used to generate the pAP-217 construct;

Figure 4B shows the nucleotide sequence of the MMP-7 linker regions of pAP-217;

10           Figure 4C shows the subcloning of the MMP-7 linker variant into a baculovirus transfer vector;

Figure 4D shows the DNA sequence of the pAP-218 insert containing ricin and the MMP-7 linker;

Figure 5A summarizes the cloning strategy used to generate the pAP-219 construct;

15           Figure 5B shows the nucleotide sequence of the MMP-9 linker regions of pAP-219;

Figure 5C shows the subcloning of the MMP-9 linker variant into a baculovirus transfer vector;

20           Figure 5D shows the DNA sequence of the pAP-220 insert containing ricin and the MMP-9 linker.

Figure 6A summarizes the cloning strategy used to generate the pAP-221 construct;

Figure 6B shows the nucleotide sequence of the thermolysin-like MMP linker regions of pAP-221;

25           Figure 6C shows the subcloning of the thermolysin-like MMP linker variant into a baculovirus transfer vector.

Figure 6D shows the DNA sequence of the pAP-222 insert containing ricin and the thermolysin-like MMP linker;

30           Figure 7A summarizes the cloning strategy used to generate the pAP-223 construct;

Figure 7B shows the nucleotide sequence of the Plasmodium falciparum-A linker regions of pAP-223;

Figure 7C shows the subcloning of the Plasmodium falciparum-A linker variant into a baculovirus transfer vector;

Figure 7D shows the DNA sequence of the pAP-224 insert containing ricin and the *Plasmodium falciparum*-A linker;

Figure 8A summarizes the cloning strategy used to generate the pAP-225 construct;

5        Figure 8B shows the nucleotide sequence of the *Plasmodium falciparum*-B linker regions of pAP-225;

Figure 8C shows the subcloning of the *Plasmodium falciparum*-B linker variant into a baculovirus transfer vector;

10       Figure 8D shows the DNA sequence of the pAP-226 insert containing ricin and the *Plasmodium falciparum*-B linker;

Figure 9A summarizes the cloning strategy used to generate the pAP-227 construct;

Figure 9B shows the nucleotide sequence of the *Plasmodium falciparum*-C linker regions of pAP-227;

15       Figure 9C shows the subcloning of the *Plasmodium falciparum*-C linker variant into a baculovirus transfer vector;

Figure 9D shows the DNA sequence of the pAP-228 insert containing ricin and the *Plasmodium falciparum*-C linker;

20       Figure 10A summarizes the cloning strategy used to generate the pAP-229 construct;

Figure 10B shows the nucleotide sequence of the *Plasmodium falciparum*-D linker regions of pAP-229;

Figure 10C shows the subcloning of the *Plasmodium falciparum*-D linker variant into a baculovirus transfer vector;

25       Figure 10D shows the DNA sequence of the pAP-230 insert containing ricin and the *Plasmodium falciparum*-D linker;

Figure 11A summarizes the cloning strategy used to generate the pAP-231 construct;

30       Figure 11B shows the nucleotide sequence of the *Plasmodium falciparum*-E linker regions of pAP-231;

Figure 11C shows the subcloning of the *Plasmodium falciparum*-E linker variant into a baculovirus transfer vector;

Figure 11D shows the DNA sequence of the pAP-232 insert containing ricin and the *Plasmodium falciparum*-E linker;

Figure 12A summarizes the cloning strategy used to generate the pAP-233 construct;

Figure 12B shows the nucleotide sequence of the HSV-A linker regions of pAP-233;

5           Figure 12C shows the subcloning of the HSV-A linker variant into a baculovirus transfer vector;

Figure 12D shows the DNA sequence of the pAP-234 insert containing ricin and the HSV-A linker;

10           Figure 13A summarizes the cloning strategy used to generate the pAP-235 construct;

Figure 13B shows the nucleotide sequence of the HSV-B linker regions of pAP-235;

Figure 13C shows the subcloning of the HSV-B linker variant into a baculovirus transfer vector;

15           Figure 13D shows the DNA sequence of the pAP-236 insert containing ricin and the HSV-B linker;

Figure 14A summarizes the cloning strategy used to generate the pAP-237 construct;

20           Figure 14B shows the nucleotide sequence of the VZV-A linker regions of pAP-237;

Figure 14C shows the subcloning of the VZV-A linker variant into a baculovirus transfer vector;

Figure 14D shows the DNA sequence of the pAP-238 insert containing ricin and the VZV-A linker;

25           Figure 15A summarizes the cloning strategy used to generate the pAP-239 construct;

Figure 15B shows the nucleotide sequence of the VZV-B linker regions of pAP-239;

30           Figure 15C shows the subcloning of the VZV-B linker variant into a baculovirus transfer vector;

Figure 15D shows the DNA sequence of the pAP-240 insert containing ricin and the VZV-B linker;

Figure 16A summarizes the cloning strategy used to generate the pAP-241 construct;

Figure 16B shows the nucleotide sequence of the EBV-A linker regions of pAP-241;

Figure 16C shows the subcloning of the EBV-A linker variant into a baculovirus transfer vector;

5        Figure 16D shows the DNA sequence of the pAP-242 insert containing ricin and the EBV-A linker;

Figure 17A summarizes the cloning strategy used to generate the pAP-243 construct;

10       Figure 17B shows the nucleotide sequence of the EBV-B linker regions of pAP-243;

Figure 17C shows the subcloning of the EBV-B linker variant into a baculovirus transfer vector;

Figure 17D shows the DNA sequence of the pAP-244 insert containing ricin and the EBV-B linker;

15       Figure 18A summarizes the cloning strategy used to generate the pAP-245 construct;

Figure 18B shows the nucleotide sequence of the CMV-A linker regions of pAP-245;

20       Figure 18C shows the subcloning of the CMV-A linker variant into a baculovirus transfer vector;

Figure 18D shows the DNA sequence of the pAP-246 insert containing ricin and the CMV-A linker;

Figure 19A summarizes the cloning strategy used to generate the pAP-247 construct;

25       Figure 19B shows the nucleotide sequence of the CMV-B linker regions of pAP-247;

Figure 19C shows the subcloning of the CMV-B linker variant into a baculovirus transfer vector;

30       Figure 19D shows the DNA sequence of the pAP-248 insert containing ricin and the CMV-B linker.

Figure 20A summarizes the cloning strategy used to generate the pAP-249 construct;

Figure 20B shows the nucleotide sequence of the HHV-6 linker regions of pAP-249;

Figure 20C shows the subcloning of the HHV-6 linker variant into a baculovirus transfer vector;

Figure 20D shows the DNA sequence of the pAP-250 insert containing ricin and the HHV-6 linker;

5           Figure 21 shows the amino acid sequences of the wild type ricin linker and cancer protease-sensitive amino acid linkers contained in pAP-213 to pAP-222 and linkers pAP-241 to pAP-244;

Figure 22A summarizes the cloning strategy used to generate the pAP-253 construct;

10           Figure 22B shows the nucleotide sequence of the ILV linker regions of pAP-253;

Figure 22C shows the subcloning of the ILV linker variant into a baculovirus transfer vector;

15           Figure 22D shows the DNA sequence of the pAP-254 insert containing ricin and the ILV linker;

Figure 23A summarizes the cloning strategy used to generate the pAP-257 construct;

Figure 23B shows the nucleotide sequence of the HAV-A linker regions of pAP-257;

20           Figure 23C shows the subcloning of the HAV-A linker variant into a baculovirus transfer vector;

Figure 23D shows the DNA sequence of the pAP-258 insert containing ricin and the HAV-A linker;

25           Figure 24A summarizes the cloning strategy used to generate the pAP-255 construct;

Figure 24B shows the nucleotide sequence of the HAV-B linker regions of pAP-255;

Figure 24C shows the subcloning of the HAV-B linker variant into a baculovirus transfer vector;

30           Figure 24D shows the DNA sequence of the pAP-256 insert containing ricin and the HAV-B linker;

Figure 25A summarizes the cloning strategy used to generate the pAP-259 construct;

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Figure 25B shows the nucleotide sequence of the CAN linker regions of pAP-259;

Figure 25C shows the subcloning of the CAN linker variant into a baculovirus transfer vector;

5           Figure 25D shows the DNA sequence of the pAP-260 insert containing ricin and the CAN linker;

Figure 26 shows the amino acid sequences of the wild type ricin linker and *Plasmodium falciparum* protease-sensitive amino acid linkers contained in linkers pAP-223 to pAP-232;

10           Figure 27 shows the amino acid sequences of the wild type ricin linker and the viral protease-sensitive amino acid linkers contained in pAP-233 to pAP-240, pAP-245-pAP-248, pAP-253 to pAP-258;

Figure 28 shows the amino acid sequences of the wild type ricin linker and the *Candida* aspartic protease-sensitive amino acid linker  
15           contained in pAP-259 to pAP-264;

Figure 29 describes an alternative mutagenesis and subcloning strategy to provide a baculovirus transfer vector containing the ricin-like toxin variant gene; and

Figure 30A summarizes the cloning strategy used to  
20           generate the pAP-262 construct;

Figure 30B shows the nucleotide sequence of the HCV-A linker region of pAP-262;

Figure 30C shows the DNA sequence of the pAP-262 insert;

Figure 30D shows the amino acid sequence comparison of  
25           mutant preproricin linker region HCV-A to wild type;

Figure 31A summarizes the cloning strategy used to generate the pAP-264 construct;

Figure 31B shows the nucleotide sequence of the HCV-B linker region of pAP-264;

30           Figure 31C shows the DNA sequence of the pAP-264 insert;

Figure 31D shows the amino acid sequence comparison of mutant preproricin linker region HCV-B to wild type;

Figure 32A summarizes the cloning strategy used to generate the pAP-266 construct;

Figure 32B shows the nucleotide sequence of the HCV-C linker region of pAP-266;

Figure 32C shows the DNA sequence of the pAP-266 insert;

Figure 32D shows the amino acid sequence comparison of  
5 mutant preproricin linker region HCV-C to wild type;

Figure 33A summarizes the cloning strategy used to generate the pAP-268 construct;

Figure 33B shows the nucleotide sequence of the HCV-D linker region of pAP-268;

10 Figure 33C shows the DNA sequence of the pAP-268 insert;

Figure 33D shows the amino acid sequence comparison of mutant preproricin linker region HCV-D to wild type;

Figure 34A summarizes the cloning strategy used to generate the pAP-270 construct;

15 Figure 34B shows the nucleotide sequence of the MMP-2 linker region of pAP-270;

Figure 34C shows the DNA sequence of the pAP-270 insert;

Figure 34D shows the amino acid sequence comparison of mutant preproricin linker region of MMP-2 to wild type;

20 Figure 35A summarizes the cloning strategy used to generate the pAP-272 construct;

Figure 35B shows the nucleotide sequence of the Cathepsin B (Site 2) linker region of pAP-272;

Figure 35C shows the DNA sequence of the pAP-272 insert;

25 Figure 35D shows the amino acid sequence comparison of mutant preproricin linker region of Cathepsin B (Site 2) to wild type;

Figure 36A summarizes the cloning strategy used to generate the pAP-274 construct;

30 Figure 36B shows the nucleotide sequence of the Cathepsin L linker region of pAP-274;

Figure 36C shows the DNA sequence of the pAP-274 insert;

Figure 36D shows the amino acid sequence comparison of mutant preproricin linker region of Cathepsin L to wild type;

Figure 37A summarizes the cloning strategy used to generate the pAP-276 construct;

Figure 37B shows the nucleotide sequence of the Cathepsin D linker region of pAP-276;

5 Figure 37C shows the DNA sequence of the pAP-276 insert;

Figure 37D shows the amino acid sequence comparison of mutant preproricin linker region of Cathepsin D to wild type;

Figure 38A summarizes the cloning strategy used to generate the pAP-278 construct;

Figure 38B shows the nucleotide sequence of the MMP-1 linker region of pAP-278;

Figure 38C shows the DNA sequence of the pAP-278 insert;

Figure 38D shows the amino acid sequence comparison of mutant preproricin linker region of MMP-1 to wild type;

Figure 39A summarizes the cloning strategy used to generate the pAP-280 construct;

Figure 39B shows the nucleotide sequence of the Urokinase-Type Plasminogen Activator linker region of pAP-280;

Figure 39C shows the DNA sequence of the pAP-280 insert;

20 Figure 39D shows the amino acid sequence comparison of mutant preproricin linker region of Urokinase-Type Plasminogen Activator to wild type;

Figure 40A summarizes the cloning strategy used to generate the pAP-282 construct;

25 Figure 40B shows the nucleotide sequence of the MT-MMP  
linker region of pAP-282;

Figure 40C shows the DNA sequence of the pAP-282 insert;

Figure 40D shows the amino acid sequence comparison of mutant preproricin linker region of MT-MMP to wild type;

Figure 41A summarizes the cloning strategy used to generate the pAP-284 construct;

Figure 41B shows the nucleotide sequence of the MMP-11 linker region of pAP-284;

Figure 41C shows the DNA sequence of the pAP-284 insert;



Figure 41D shows the amino acid sequence comparison of mutant preprorin linker region of MMP-11 to wild type;

Figure 42A summarizes the cloning strategy used to generate the pAP-286 construct;

5        Figure 42B shows the nucleotide sequence of the MMP-13 linker region of pAP-286;

Figure 42C shows the DNA sequence of the pAP-286 insert;

Figure 42D shows the amino acid sequence comparison of mutant preprorin linker region of MMP-13 to wild type;

10       Figure 43A summarizes the cloning strategy used to generate the pAP-288 construct;

Figure 43B shows the nucleotide sequence of the Tissue-type Plasminogen Activator linker region of pAP-288;

Figure 43C shows the DNA sequence of the pAP-288 insert;

15       Figure 43D shows the amino acid sequence comparison of mutant preprorin linker region of Tissue-type Plasminogen Activator to wild type;

Figure 44A summarizes the cloning strategy used to generate the pAP-290 construct;

20       Figure 44B shows the nucleotide sequence of the human Prostate-Specific Antigen linker region of pAP-290;

Figure 44C shows the DNA sequence of the pAP-290 insert;

Figure 44D shows the amino acid sequence comparison of mutant preprorin linker region of the human Prostate-Specific Antigen to  
25       wild type;

Figure 45A summarizes the cloning strategy used to generate the pAP-292 construct;

Figure 45B shows the nucleotide sequence of the kallikrein linker region of pAP-292;

30       Figure 45C shows the DNA sequence of the pAP-292 insert;

Figure 45D shows the amino acid sequence comparison of mutant preprorin linker region of the kallikrein to wild type;

Figure 46A summarizes the cloning strategy used to generate the pAP-294 construct;

Figure 46B shows the nucleotide sequence of the neutrophil elastase linker region of pAP-294;

Figure 46C shows the DNA sequence of the pAP-294 insert;

Figure 46D shows the amino acid sequence comparison of  
5 mutant preproricin linker region of neutrophil elastase to wild type;

Figure 47A summarizes the cloning strategy used to generate the pAP-296 construct;

Figure 47B shows the nucleotide sequence of the calpain linker region of pAP-296;

10 Figure 47C shows the DNA sequence of the pAP-296 insert;

Figure 47D shows the amino acid sequence comparison of mutant preproricin linker region of calpain to wild type;

Figure 48 is a blot showing cleavage of pAP-214 by  
Cathepsin B;

15 Figure 49 is a blot showing cleavage of pAP-220 with  
MMP-9;

Figure 50 is a blot showing activation of pAP-214; and

Figure 51 is a blot showing activation of pAP-220.

Figure 52 is a blot showing cleavage of pAP-248 with  
20 HCMV.

Figure 53 is a blot showing activation of pAP-248.

Figure 54 is a blot showing cleavage of pAP-256 by HAV  
3C.

Figure 55 is a blot showing activation of pAP-256.

25 Figure 56 is a semi-logithmic graph illustrating the  
cytotoxicity to COS-1 cells of undigested pAP-214 and pAP-214  
digested with Cathepsin B.

Figure 57 is a semi-logithmic graph illustrating the  
cytotoxicity of pAP-220 digested with MMP-9 compared to freshly  
30 thawed pAP-220 and ricin on COS-1 cells.

Figure 58 is a blot showing cleavage of pAP-270 with  
MMP-2.

Figure 59 is a blot showing activation of pAP-270.

Figure 60 is a blot showing cleavage of pAP-288 by t-PA.

Figure 61 is a blot showing activation of pAP-288.

Figure 62 is a blot showing cleavage of pAP-294 with human neutrophil elastase.

Figure 63 is a blot showing activation of pAP-294.

5 Figure 64 is a blot showing cleavage of pAP-296 with calpain.

Figure 65 is a blot showing activation of pAP-296.

Figure 66 is a blot showing cleavage of pAP-222 with MMP-2.

10 Figure 67 is a blot showing activation of pAP-222.

## **DETAILED DESCRIPTION OF THE INVENTION**

### **Nucleic Acid Molecules of the Invention**

As mentioned above, the present invention relates to novel nucleic acid molecules comprising a nucleotide sequence encoding an A chain of a ricin-like toxin, a B chain of a ricin-like toxin and a heterologous linker amino acid sequence, linking the A and B chains. The heterologous linker sequence contains a cleavage recognition site for a disease-specific protease (e.g. a viral protease, parasitic protease, cancer-associated protease, or a fungal protease).

20 The term "isolated and purified" as used herein refers to a nucleic acid substantially free of cellular material or culture medium when produced by recombinant DNA techniques, or chemical precursors, or other chemicals when chemically synthesized. An "isolated and purified" nucleic acid is also substantially free of sequences which naturally flank the nucleic acid (*i.e.* sequences located at the 5' and 3' ends of the nucleic acid) from which the nucleic acid is derived. The term "nucleic acid" is intended to include DNA and RNA and can be either double stranded or single stranded.

30 The term "linker sequence" as used herein refers to an internal amino acid sequence within the protein encoded by the nucleic acid molecule of the invention which contains residues linking the A and B chain so as to render the A chain incapable of exerting its toxic effect, for example catalytically inhibiting translation of a eukaryotic ribosome. By heterologous is meant that the linker sequence is not a sequence native to

the A or B chain of a ricin-like toxin or precursor thereof. However, preferably, the linker sequence may be of a similar length to the linker sequence of a ricin-like toxin and should not interfere with the role of the B chain in cell binding and transport into the cytoplasm. When the linker  
5 sequence is cleaved the A chain becomes active or toxic.

The nucleic acid molecule of the invention is cloned by subjecting a preproricin cDNA clone to site-directed mutagenesis in order to generate a series of variants differing only in the sequence between the A and B chains (linker region). Oligonucleotides, corresponding to the  
10 extreme 5' and 3' ends of the preproricin gene are synthesized and used to PCR amplify the gene. Using the cDNA sequence for preproricin (Lamb et al., *Eur. J. Biochem.* 145:266-270 (1985)), several oligonucleotide primers are designed to flank the start and stop codons of the preproricin open reading frame.

The preproricin cDNA is amplified using the upstream primer Ricin-99 or Ricin-109 and the downstream primer Ricin1729C with Vent DNA polymerase (New England Biolabs) using standard procedures (Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Second Edition, (Cold Spring Harbor Laboratory Press, 1989)). The purified PCR  
20 fragment encoding the preproricin cDNA is then ligated into an Eco RI-digested pBluescript II SK plasmid (Stratagene), and is used to transform competent XL1-Blue cells (Stratagene). The cloned PCR product containing the putative preproricin gene is confirmed by DNA sequencing of the entire cDNA clone. The sequences and location of oligonucleotide  
25 primers used for sequencing are shown in Table 1.

The preproricin cDNA clone is subjected to site directed mutagenesis in order to generate a series of variants differing only in the sequence between the A and B chains (linker region). The wild-type preproricin linker region is replaced with the heterogenous linker  
30 sequences that are cleaved by the various disease-specific proteases as shown in Figures 21, 26, 27, 28, and Part D of Figures 30-47. Linker identification as used herein in connection with the sequences provided in these figures have been assigned the sequence ID numbers as discussed below.

The linker regions of the variants encode a cleavage recognition sequence for a disease-specific protease associated with for example, cancer, viruses, parasites, or fungi. The mutagenesis and cloning strategy used to generate the disease-specific protease-sensitive linker variants are summarized in Part A of Figures 2-20, and Part A of Figures 22-25. The first step involves a DNA amplification using a set of mutagenic primers in combination with the two flanking primers Richin-99Eco or Ricin-109Eco and Ricin1729C Pst I. Restriction digested PCR fragments are gel purified and then ligated with PBluescript SK which has been digested with Eco RI and Pst I. Ligation reactions are used to transform competent XL1-Blue cells (Stratagene). Recombinant clones are identified by restriction digests of plasmid miniprep DNA and the mutant linker sequences are confirmed by DNA sequencing. With respect to the nucleotide sequences and amino acid sequences prepared as a result of the implementation of this strategy the following sequences have been assigned the sequence ID numbers as indicated.

SEQ ID NO. 1 is used herein in connection with the DNA sequence of the baculovirus transfer vector, pVL1393.

The nucleotide sequence of Cathepsin B linker regions of pAP-213 are referred to herein as SEQ ID NO. 2.

The nucleotide sequence of Cathepsin B linker regions of pAP-214 are referred to herein as SEQ ID NO. 3.

The nucleotide sequence of MMP-3 linker regions of pAP-215 are referred to herein as SEQ ID NO. 4.

The DNA sequence of the pAP-216 insert containing ricin and the MMP-3 linker are referred to herein as SEQ ID NO. 5.

The nucleotide sequence of MMP-7 linker regions of pAP-217 are referred to herein as SEQ ID NO. 6.

The DNA sequence of the pAP-218 insert containing ricin and the MMP-7 linker are referred to herein as SEQ ID NO. 7.

The nucleotide sequence of MMP-9 linker regions of pAP-219 are referred to herein as SEQ ID NO. 8.

The DNA sequence of the pAP-220 insert containing ricin and the MMP-9 are referred to herein as SEQ ID NO. 9.

The nucleotide sequence of thermolysin-like MMP linker regions of pAP-221 are referred to herein as SEQ ID NO. 10.

The DNA sequence of of pAP-222 insert containing ricin and the thermolysin-like MMP linker are referred to herein as SEQ ID NO. 11.

5 The nucleotide sequence of Plasmodium falciparum-A linker regions of pAP-223 are referred to herein as SEQ ID NO. 12.

The DNA sequence of the pAP-224 insert containing ricin and the Plasmodium falciparum-A linker are referred to herein as SEQ ID NO. 13.

10 The nucleotide sequence of Plasmodium falciparum-B linker regions of pAP-225 are referred to herein as SEQ ID NO. 14.

The DNA sequence of the pAP-226 insert containing ricin and the Plasmodium falciparum-B linker are referred to herein as SEQ ID NO. 15.

15 The nucleotide sequence of Plasmodium falciparum-C linker regions of pAP-227 are referred to herein as SEQ ID NO. 16.

The DNA sequence of the pAP-228 insert containing ricin and the Plasmodium falciparum-C linker are referred to herein as SEQ ID NO. 17.

20 The nucleotide sequence of the the Plasmodium falciparum-D linker regions of pAP-229 is referred to herein as SEQ ID NO. 18.

The DNA sequence of the pAP-230 insert containing ricin and the Plasmodium falciparum-D linker is referred to herein as SEQ ID NO. 19.

25 The nucleotide sequence of the Plasmodium falciparum-E linker regions of pAP-231 is referred to herein as SEQ ID NO. 20.

The DNA sequence of the pAP-232 insert containing ricin and the Plasmodium falciparum-E linker is referred to herein as SEQ ID NO. 21.

30 The nucleotide sequence of the HSV-A linker regions of pAP-233 is referred to herein as SEQ ID NO. 22.

The DNA sequence of the pAP-234 insert containing ricin and the HSV-A linker is referred to herein as SEQ ID NO. 23.

The nucleotide sequence of the HSV-B linker regions of pAP-235 is referred to herein as SEQ ID NO. 24.

The DNA sequence of the pAP-236 insert containing ricin and the HSV-B linker is referred to herein as SEQ ID NO. 25.

5 The nucleotide sequence of the VZV-A linker regions of pAP-237 are referred to herein as SEQ ID NO. 26.

The DNA sequence of the pAP-238 insert containing ricin and the VZV-A linker are referred to herein as SEQ ID NO. 27.

10 The nucleotide sequence of the VZV-B linker regions of PAP-239 is referred to herein as SEQ ID NO. 28.

The DNA sequence of the pAP-240 insert containing ricin and the VZV-B linker is referred to herein as SEQ ID NO. 29.

The nucleotide sequence of the EBV-A linker regions of pAP-241 is referred to herein as SEQ ID NO. 30.

15 The DNA sequence of the pAP-242 insert containing ricin and the EBV-A linker is referred to herein as SEQ ID NO. 31.

The nucleotide sequence of the EBV-B linker regions of pAP-243 is referred to herein as SEQ ID NO. 32.

20 The DNA sequence of the pAP-244 insert containing ricin and the EBV-B linker is referred to herein as SEQ ID NO. 33.

The nucleotide sequence of the CMV-A linker regions of pAP-245 is referred to herein as SEQ ID NO. 34.

The DNA sequence of the pAP-246 insert containing ricin and the CMV-A linker is referred to herein as SEQ ID NO. 35.

25 The nucleotide sequence of the CMV-B linker regions of pAP-247 is referred to herein as SEQ ID NO. 36.

The DNA sequence of the pAP-248 insert containing ricin and the CMV-B linker is referred to herein as SEQ ID NO. 37.

30 The nucleotide sequence of the HHV-6 linker regions of pAP-249 is referred to herein as SEQ ID NO. 38.

The DNA sequence of the pAP-250 insert containing ricin and the HHV-6 linker is referred to herein as SEQ ID NO. 39.

The amino acid sequences of the cancer protease-sensitive amino acid linkers contained in the following pAP proteins have the

sequence ID numbers as indicated: pAP-213 and pAP-214 (SEQ ID NO. 40); pAP-215 and pAP-216 (SEQ ID NO. 41); pAP-217 and pAP-218; (SEQ ID NO. 42); pAP-219 and pAP-220 (SEQ ID NO. 43); and pAP-221 and pAP-222 (SEQ ID NO. 44).

5           The amino acid sequences of the following cancer protease-sensitive linkers are referred to herein with the corresponding sequence ID numbers: pAP-241 and pAP-242 (SEQ ID NO. 45); and pAP-243 and pAP-244 (SEQ ID NO. 46).

10           The nucleotide sequence of the ILV linker regions of pAP-253 is referred to herein as SEQ ID NO. 47.

          The DNA sequence of the pAP-254 insert containing ricin and the ILV linker is referred to herein as SEQ ID NO. 48.

          The nucleotide sequence of the HAV-A linker regions of pAP-257 is referred to herein as SEQ ID NO. 49.

15           The DNA sequence of the pAP-258 insert containing ricin and HAV-A linker is referred to herein as SEQ ID NO. 50.

          The nucleotide sequence of the HAV-B linker regions of pAP-255 is referred to herein as SEQ ID NO. 51.

20           The DNA sequence of the pAP-256 insert containing ricin and the HAV-B linker is referred to herein as SEQ ID NO. 52.

          The nucleotide sequence of the CAN linker regions of pAP-259 is referred to herein as SEQ ID NO. 53.

          The DNA sequence of the pAP-260 insert containing ricin and the CAN linker is referred to herein as SEQ ID NO. 54.

25           The amino acid sequences of *Plasmodium falciparum* protease-sensitive linkers are referred to herein by the sequence ID numbers as follows: pAP-223 and pAP-224 (SEQ ID NO 55); pAP-225 and pAP-226 (SEQ ID NO 56); pAP-227 and pAP-228 (SEQ ID NO 57); pAP-229 and pAP-230 (SEQ ID NO 58); and pAP-231 and pAP-232 (SEQ ID NO 59)  
30 (see Figure 26).

          The amino acid sequences of the viral protease-sensitive linkers which follow are referred to herein by the sequence ID numbers indicated: pAP-233 and pAP 234 (SEQ ID NO 60); pAP-235 and pAP-236 (SEQ ID NO 61); and pAP-249 and pAP-250 (SEQ ID NO 62) (see Figure 27).



5 The amino acid sequences of the viral protease-sensitive  
linkers which follow are referred to herein by the sequence ID numbers  
indicated: pAP-237 and pAP-238 (SEQ ID NO 65); and pAP-239 and pAP-  
240 (SEQ ID NO 66); pAP-253 and pAP-254 (SEQ ID NO 67); pAP-255 and  
pAP-256 (SEQ ID NO 68); and pAP-257 and pAP-258 (SEQ ID NO 69) (see  
10 Figure 27).

An alternative mutagenesis and cloning strategy that can be used to generate the disease-specific protease-sensitive linker variants is summarized in Figure 29. The first step of this method involves a DNA amplification using a set of mutagenic primers in combination with the two flanking primers Ricin-109Eco and Ricin1729Pst. Restriction digested PCR fragments (Eco RI and Pst I) are gel purified. Preproricin variants produced from this method can be subcloned directly into the baculovirus transfer vector digested with Eco RI and Pst I and intermediate ligation steps involving pBluescript SK and pSB2 are circumvented. The cloning strategies used to generate disease-specific protease-sensitive linker variants are summarized in Part A of Figures 30 to 47. With respect to the nucleotide sequences and amino acid sequences prepared as a result of the implementation of this strategy the following sequences have been assigned the sequence ID numbers as indicated.

The DNA sequence of the pAP-262 insert is referred to herein as SEQ ID NO. 74.

The amino acid sequence of the mutant preproricin linker region for HCV-A, pAP-262, is referred to herein as SEQ ID NO. 75.

The nucleotide sequence of the HCV-B linker region of pAP-264 is referred to herein as SEQ ID NO. 76.

The DNA sequence of the pAP-264 insert is referred to herein as SEQ ID NO. 77.

5           The amino acid sequence of the mutant preproricin linker region for HCV-B, pAP-264, is referred to herein as SEQ ID NO. 78.

The nucleotide sequence of the HCV-C linker region of pAP-266 is referred to herein as SEQ ID NO. 79.

10           The DNA sequence of the pAP-266 insert is referred to herein as SEQ ID NO. 80.

The amino acid sequence of the mutant preproricin linker region for HCV-C, pAP-266, is referred to herein as SEQ ID NO. 81.

The nucleotide sequence of the HCV-D linker region of pAP-268 is referred to herein as SEQ ID NO. 82.

15           The DNA sequence of the pAP-268 insert is referred to herein as SEQ ID NO. 83.

The amino acid sequence of the mutant preproricin linker region for HCV-D , pAP-268, is referred to herein as SEQ ID NO. 84.

20           The nucleotide sequence of the MMP-2 linker region of pAP-270 is referred to herein as SEQ ID NO. 85.

The DNA sequence of the pAP-270 insert is referred to herein as SEQ ID NO. 86.

The amino acid sequence of the mutant preproricin linker region for MMP-2, pAP-270, is referred to herein as SEQ ID NO. 87.

25           The nucleotide acid sequence of the Cathepsin B (Site 2) linker region of pAP-272 is referred to herein as SEQ ID NO. 88.

The DNA sequence of the pAP-272 insert is referred to herein as SEQ ID NO. 89.

30           The amino acid sequence of the mutant preproricin linker region for Cathepsin B (Site 2), pAP-272, is referred to herein as SEQ ID NO. 90.

The nucleotide sequence of the Cathepsin L linker region of pAP-274 is referred to herein as SEQ ID NO. 91.

The DNA sequence of the pAP-274 insert is referred to herein as SEQ ID NO. 92.

The amino acid sequence of the mutant preproricin linker region of Cathepsin L, pAP-274, is referred to herein as SEQ ID NO. 93.

5 The nucleotide sequence of Cathepsin D linker region of pAP-276 is referred to herein as SEQ ID NO. 94.

The DNA sequence of the pAP-276 insert is referred to herein as SEQ ID NO. 95.

10 The amino acid sequence of the mutant preproricin linker region for Cathepsin D, pAP-276, is referred to herein as SEQ ID NO. 96.

The nucleotide sequence of the MMP-1 linker region of pAP-278 is referred to herein as SEQ ID NO. 97.

The DNA sequence of the pAP-278 insert is referred to herein as SEQ ID NO. 98.

15 The amino acid sequence of the mutant preproricin linker region for MMP-1, pAP-278, is referred to herein as SEQ ID NO. 99.

The nucleotide sequence of the Urokinase-Type Plasminogen Activator linker region of pAP-280 is referred to herein as SEQ ID NO. 100.

20 The DNA sequene of the pAP-280 insert is referred to herein as SEQ ID NO. 101.

The amino acid sequence of the mutant preproricin linker region for Urokinase-Type Plasminogen Activator, pAP-280, is referred to herein as SEQ ID NO. 102.

25 The nucleotide sequence of MT-MMP linker region of pAP-282 is referred to herein as SEQ ID NO. 103.

The DNA sequence of the pAP-282 insert is referred to herein as SEQ ID NO. 104.

30 The amino acid sequence of the mutant preproricin linker region for MT-MMP, pAP-282, is referred to herein as SEQ ID NO. 105.

The nucleotide sequence of the MMP-11 linker region of pAP-284 is referred to herein as SEQ ID NO. 106.

The DNA sequence of the pAP-284 insert is referred to herein as SEQ ID NO. 107.

5           The DNA sequence of the pAP-286 insert is referred to  
herein as SEQ ID NO. 110.

The amino acid sequence of the mutant preproricin linker region for MMP-13, pAP-286, is referred to herein as SEQ ID NO. 111.

The DNA sequence of the pAP-288 insert is referred to herein as SEQ ID NO. 113.

The nucleotide sequence of the human Prostate-Specific Antigen linker region of pAP-290 is referred to herein as SEQ ID NO. 115.

20           The amino acid sequence of the mutant preproricin linker region for the human Prostate-Specific Antigen, pAP-290, is referred to herein as SEO ID NO. 117.

25                   The DNA sequence of the pAP-292 insert is referred to  
herein as SEQ ID NO. 119.

The nucleotide sequence of the neutrophil elastase linker  
30 region of pAP-294 is referred to herein as SEQ ID NO. 121.

The DNA sequence of the pAP-294 insert is referred to herein as SEQ ID NO. 122.

The amino acid sequence of the mutant preproricin linker region for neutrophil elastase, pAP-294, is referred to herein as SEQ ID NO. 123.

5 The nucleotide sequence of the calpain linker region of pAP-296 is referred to herein as SEQ ID NO. 124.

The DNA sequence of the pAP-296 insert is referred to herein as SEQ ID NO. 125.

The amino acid sequence of the mutant preproricin linker region for calpain, pAP-296, is referred to herein as SEQ ID NO. 126.

10 The amino acid sequence of the wild type linker region is referred to herein as SEQ ID NO. 127.

The nucleic acid molecule of the invention has sequences encoding an A chain of a ricin-like toxin, a B chain of a ricin-like toxin and a heterologous linker sequence containing a cleavage recognition site for a disease-specific protease. The nucleic acid may be expressed to provide a recombinant protein having an A chain of a ricin-like toxin, a B chain of a ricin-like toxin and a heterologous linker sequence containing a cleavage recognition site for a disease-specific protease.

20 The nucleic acid molecule may comprise the A and/or B chain of ricin. The ricin gene has been cloned and sequenced, and the X-ray crystal structures of the A and B chains are published (Rutenber, E., et al. *Proteins* 10:240-250 (1991); Weston et al., *Mol. Biol.* 244:410-422 (1994); Lamb and Lord, *Eur. J. Biochem.* 14:265 (1985); Halling, K., et al., *Nucleic Acids Res.* 13:8019 (1985)). It will be appreciated that the invention includes nucleic acid molecules encoding truncations of A and B chains of ricin like proteins and analogs and homologs of A and B chains of ricin-like proteins and truncations thereof (i.e., ricin-like proteins), as described herein. It will further be appreciated that variant forms of the nucleic acid molecules of the invention which arise by alternative splicing of an mRNA corresponding to a cDNA of the invention are encompassed by the invention.

30 Another aspect of the invention provides a nucleotide sequence which hybridizes under high stringency conditions to a nucleotide sequence encoding the A and/or B chains of a ricin-like protein.

Appropriate stringency conditions which promote DNA hybridization are known to those skilled in the art, or can be found in Current Protocols in Molecular Biology, John Wiley & Sons, N.Y. (1989), 6.3.1 6.3.6. For example, 6.0 x sodium chloride/sodium citrate (SSC) at about 45°C, followed by a wash of 2.0 x SSC at 50°C may be employed. The stringency may be selected based on the conditions used in the wash step. By way of example, the salt concentration in the wash step can be selected from a high stringency of about 0.2 x SSC at 50°C. In addition, the temperature in the wash step can be at high stringency conditions, at about 65°C.

The nucleic acid molecule may comprise the A and/or B chain of a ricin-like toxin. Methods for cloning ricin-like toxins are known in the art and are described, for example, in E.P. 466,222. Sequences encoding ricin or ricin-like A and B chains may be obtained by selective amplification of a coding region, using sets of degenerative primers or probes for selectively amplifying the coding region in a genomic or cDNA library. Appropriate primers may be selected from the nucleic acid sequence of A and B chains of ricin or ricin-like toxins. It is also possible to design synthetic oligonucleotide primers from the nucleotide sequences for use in PCR. Suitable primers may be selected from the sequences encoding regions of ricin-like proteins which are highly conserved, as described for example in U.S. Patent No 5,101,025 and E.P. 466,222.

A nucleic acid can be amplified from cDNA or genomic DNA using these oligonucleotide primers and standard PCR amplification techniques. The nucleic acid so amplified can be cloned into an appropriate vector and characterized by DNA sequence analysis. It will be appreciated that cDNA may be prepared from mRNA, by isolating total cellular mRNA by a variety of techniques, for example, by using the guanidinium-thiocyanate extraction procedure of Chirgwin et al., *Biochemistry* 18, 5294-5299 (1979). cDNA is then synthesized from the mRNA using reverse transcriptase (for example, Moloney MLV reverse transcriptase available from Gibco/BRL, Bethesda, MD, or AMV reverse transcriptase available from Seikagaku America, Inc., St. Petersburg, FL). It will be appreciated that the methods described above may be used to obtain the coding sequence from plants, bacteria or fungi, preferably

plants, which produce known ricin-like proteins and also to screen for the presence of genes encoding as yet unknown ricin-like proteins.

A sequence containing a cleavage recognition site for a specific protease may be selected based on the disease or the pathogen which is to be targeted by the recombinant protein. The cleavage recognition site may be selected from sequences known to encode a cleavage recognition site for the cancer, viral or parasitic protease. Sequences encoding cleavage recognition sites may be identified by testing the expression product of the sequence for susceptibility to cleavage by the respective protease.

A sequence containing a cleavage recognition site for a viral, fungal, parasitic or cancer associated protease may be selected based on the retrovirus which is to be targeted by the recombinant protein. The cleavage recognition site may be selected from sequences known to encode a cleavage recognition site for the viral, fungal, parasitic or cancer associated protease. Sequences encoding cleavage recognition sites may be identified by testing the expression product of the sequence for susceptibility to cleavage by a viral, fungal, parasitic or cancer associated protease. A polypeptide containing the suspected cleavage recognition site may be incubated with a protease and the amount of cleavage product determined (DiIannit, 1990, J. Biol. Chem. 285: 17345-17354 (1990)).

The protease may be prepared by methods known in the art and used to test suspected cleavage recognition sites.

In one embodiment, the preparation of tumour-associated cathepsin B, its substrates and enzymatic activity assay methodology have been described by Sloane, B.F. et al. (*Proc. Natl. Acad. Sci. USA* 83:2483-2487 (1986)), Schwartz, M.K. (*Clin. Chim. Acta* 237:67-78 (1995)), and Panchal, R.G. et al. (*Nature Biotechnol.* 14:852-856 (1996)). The preparation of Epstein-Barr virus protease, its substrates and enzymatic activity assay methodology have been described by Welch, A.R. (*Proc. Natl. Acad. Sci. USA* 88:10792-10796 (1991)).

In another embodiment, the preparation of *Plasmodium falciparum* proteases, their substrates and enzymatic activity assay methodology have been described by Goldberg, D.E. et al. (*J. Exp. Med.*

173:961-969 (1991)), Cooper & Bujard (*Mol. Biochem. Parasitol.* 56:151-160 (1992)), Nwagwu, M. et al. (*Exp. Parasitol.* 75:399-414 (1992)), Rosenthal, P.J. et al. (*J. Clin. Invest.* 91:1052-1056 (1993)), Blackman, M.J. et al. (*Mol. Biochem. Parasitol.* 62:103-114 (1995)).

5 In a further embodiment, the preparation of proteases from human cytomegalovirus, human herpes virus, varicella zoster virus and infectious laryngotracheitis virus have been taught by Liu F. & Roizman, B. (*J. Virol.* 65:5149-5156 (1991)) and Welch, A.R. (*Proc. Natl. Acad. Sci. USA* 88:10792-10796 (1991)). In addition, their respective substrates and  
10 enzymatic activity assay methodologies are also described.

In another embodiment, the preparation of hepatitis A virus protease, its substrates and enzymatic activity assay methodology have been described by Jewell, D.A. et al. (*Biochemistry* 31:7862-7869 (1992)). The preparation of poliovirus protease, its substrates and enzymatic activity  
15 assay methodology have been described by Weidner, J.R. et al. (*Arch. Biochem. Biophys.* 286:402-408 (1991)). The preparation of human rhinovirus protease, its substrates and enzymatic activity assay methodology have been described by Long, A.C. et al. (*FEBS Lett.* 258:75-78 (1989)).

In another embodiment of the invention, the preparation of  
20 proteases associated with *Candida* yeasts their substrates and enzymatic activity are contemplated, including the aspartic proteinases which have been associated specifically with numerous virulent strains of *Candida* including *Candida albican*, *Candida tropicalis*, and *Candida parapsilosis* (Abad-Zapatero, C. et al., *Protein Sci.* 5:640-652 (1996); Cutfield, S.M. et al.,  
25 *Biochemistry* 35:398-410 (1995); Ruchel, R. et al, *Zentralbl. Bakteriolog. Mikrobiol Hyg. I Abt. Orig. A.* 255:537-548 (1983); Remold, H. et al., *Biochim. Biophys. Acta* 167:399-406 (1968)).

The nucleic acid molecule of the invention may be prepared by site directed mutagenesis. For example, the cleavage site of a disease-  
30 specific protease may be prepared by site directed mutagenesis of the homologous linker sequence of a proricin-like toxin. Procedures for cloning proricin-like genes, encoding a linker sequence are described in EP 466,222. Site directed mutagenesis may be accomplished by DNA amplification of mutagenic primers in combination with flanking primers.



Suitable procedures using the mutagenic primers are shown in Parts A and B of Figures 1-4, Figures 13-16, Figures 18-36, Figures 38-41, and Figures 50-67.

5 The nucleic acid molecule of the invention may also encode  
a fusion protein. A sequence encoding a heterologous linker sequence  
containing a cleavage recognition site for a disease-specific protease may  
be cloned from a cDNA or genomic library or chemically synthesized  
based on the known sequence of such cleavage sites. The heterologous  
linker sequence may then be fused in frame with the sequences encoding  
10 the A and B chains of the ricin-like toxin for expression as a fusion protein.  
It will be appreciated that a nucleic acid molecule encoding a fusion protein  
may contain a sequence encoding an A chain and a B chain from the same  
ricin-like toxin or the encoded A and B chains may be from different toxins.  
For example, the A chain may be derived from ricin and the B chain may  
15 be derived from abrin. A protein may also be prepared by chemical  
conjugation of the A and B chains and linker sequence using conventional  
coupling agents for covalent attachment.

An isolated and purified nucleic acid molecule of the  
invention which is RNA can be isolated by cloning a cDNA encoding an A  
20 and B chain and a linker into an appropriate vector which allows for  
transcription of the cDNA to produce an RNA molecule which encodes a  
protein of the invention. For example, a cDNA can be cloned downstream  
of a bacteriophage promoter, (e.g. a T7 promoter) in a vector, cDNA can  
be transcribed in vitro with T7 polymerase, and the resultant RNA can be  
25 isolated by standard techniques.

#### **Recombinant Protein of the Invention**

As previously mentioned, the invention provides novel  
recombinant proteins which incorporate the A and B chains of a ricin like  
toxin linked by a heterologous linker sequence containing a cleavage  
30 recognition site for a disease-specific protease. It is an advantage of the  
recombinant proteins of the invention that they are non-toxic until the A  
chain is liberated from the B chain by specific cleavage of the linker by the  
target protease.

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Thus the protein may be used to specifically target cancer cells or cells infected with a virus or parasite in the absence of additional specific cell-binding components to target infected cells. It is a further advantage that the disease-specific protease cleaves the heterologous linker intracellularly thereby releasing the toxic A chain directly into the cytoplasm of the cancer cell or infected cell. As a result, said cells are specifically targeted and non-infected normal cells are not directly exposed to the activated free A chain.

Ricin is a plant derived ribosome inhibiting protein which blocks protein synthesis in eukaryotic cells. Ricin may be derived from the seeds of *Ricinus communis* (castor oil plant). The ricin toxin is a glycosylated heterodimer with A and B chain molecular masses of 30,625 Da and 31,431 Da respectively. The A chain of ricin has an N-glycosidase activity and catalyzes the excision of a specific adenine residue from the 28S rRNA of eukaryotic ribosomes (Endo, Y; & Tsurugi, K. J. Biol. Chem. 262:8128 (1987)). The B chain of ricin, although not toxic in itself, promotes the toxicity of the A chain by binding to galactose residues on the surface of eukaryotic cells and stimulating receptor-mediated endocytosis of the toxin molecule (Simmons et al., *Biol. Chem.* 261:7912 (1986)).

All protein toxins are initially produced in an inactive, precursor form. Ricin is initially produced as a single polypeptide (preproricin) with a 35 amino acid N-terminal presequence and 12 amino acid linker between the A and B chains. The pre-sequence is removed during translocation of the ricin precursor into the endoplasmic reticulum (Lord, J.M., *Eur. J. Biochem.* 146:403-409 (1985) and Lord, J.M., *Eur. J. Biochem.* 146:411-416 (1985)). The proricin is then translocated into specialized organelles called protein bodies where a plant protease cleaves the protein at a linker region between the A and B chains (Lord, J.M. et al., *FASAB Journal* 8:201-208 (1994)). The two chains, however, remain covalently attached by an interchain disulfide bond (cysteine 259 in the A chain to cysteine 4 in the B chain) and mature disulfide linked ricin is stored in protein bodies inside plant cells. The A chain is inactive in the proricin (O'Hare, M., et al., *FEBS Lett.* 273:200-204 (1990)) and it is inactive in the disulfide-linked mature ricin (Richardson, P.T. et al., *FEBS Lett.* 255:15-20

(1989)). The ribosomes of the castor bean plant are themselves susceptible to inactivation by ricin A chain; however, as there is no cell surface galactose to permit B chain recognition the A chain cannot re-enter the cell.

Ricin-like proteins include, but are not limited to, bacterial, 5 fungal and plant toxins which have A and B chains and inactivate ribosomes and inhibit protein synthesis. The A chain is an active polypeptide subunit which is responsible for the pharmacologic effect of the toxin. In most cases the active component of the A chain is an enzyme. The B chain is responsible for binding the toxin to the cell surface and is 10 thought to facilitate entry of the A chain into the cell cytoplasm. The A and B chains in the mature toxins are linked by disulfide bonds. The toxins most similar in structure to ricin are plant toxins which have one A chain and one B chain. Examples of such toxins include abrin which may be isolated from the seeds of *Abrus precatorius*, modeccin, volkensin and 15 viscumin.

Ricin-like bacterial proteins include diphtheria toxin, which is produced by *Corynebacterium diphtheriae*, *Pseudomonas enterotoxin A* and cholera toxin. It will be appreciated that the term ricin-like toxins is also intended to include the A chain of those toxins which have only an A 20 chain. The recombinant proteins of the invention could include the A chain of these toxins conjugated to, or expressed as, a recombinant protein with the B chain of another toxin. Examples of plant toxins having only an A chain include trichosanthin, MMC and pokeweed antiviral proteins, dianthin 30, dianthin 32, croton II, curcin II and wheat germ inhibitor. 25 Examples of fungal toxins having only an A chain include alpha-sarcin, restrictocin, mitogillin, enomycin, phenomycin. Examples of bacterial toxins having only an A chain include cytotoxin from *Shigella dysenteriae* and related Shiga-like toxins. Recombinant trichosanthin and the coding sequence thereof is disclosed in U.S. Patents 5,101,025 and 5,128,460.

30 In addition to the entire A or B chains of a ricin-like toxin, it will be appreciated that the recombinant protein of the invention may contain only that portion of the A chain which is necessary for exerting its cytotoxic effect. For example, the first 30 amino acids of the ricin A chain may be removed resulting in a truncated A chain which retains toxic

activity. The truncated ricin or ricin-like A chain may be prepared by expression of a truncated gene or by proteolytic degradation, for example with Nagarase (Funmatsu et al., *Jap. J. Med. Sci. Biol.* 23:264-267 (1970)). Similarly, the recombinant protein of the invention may contain only that

5 portion of the B chain necessary for galactose recognition, cell binding and transport into the cell cytoplasm. Truncated B chains are described for example in E.P. 145,111. The A and B chains may be glycosylated or non-glycosylated. Glycosylated A and B chains may be obtained by expression in the appropriate host cell capable of glycosylation.

10 Non-glycosylated chains may be obtained by expression in nonglycosylating host cells or by treatment to remove or destroy the carbohydrate moieties.

The proteins of the invention may be prepared using recombinant DNA methods. Accordingly, the nucleic acid molecules of the

15 present invention may be incorporated in a known manner into an appropriate expression vector which ensures good expression of the protein. Possible expression vectors include but are not limited to cosmids, plasmids, or modified viruses (e.g. replication defective retroviruses, adenoviruses and adeno-associated viruses), so long as the vector is

20 compatible with the host cell used. The expression vectors are "suitable for transformation of a host cell", which means that the expression vectors contain a nucleic acid molecule of the invention and regulatory sequences selected on the basis of the host cells to be used for expression, which is operatively linked to the nucleic acid molecule. Operatively linked is

25 intended to mean that the nucleic acid is linked to regulatory sequences in a manner which allows expression of the nucleic acid.

The invention therefore contemplates a recombinant expression vector of the invention containing a nucleic acid molecule of the invention, or a fragment thereof, and the necessary regulatory sequences

30 for the transcription and translation of the inserted protein-sequence.

Suitable regulatory sequences may be derived from a variety of sources, including bacterial, fungal, viral, mammalian, or insect genes (For example, see the regulatory sequences described in Goeddel, *Gene Expression Technology: Methods in Enzymology* 185, Academic

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Press, San Diego, CA (1990). Selection of appropriate regulatory sequences is dependent on the host cell chosen as discussed below, and may be readily accomplished by one of ordinary skill in the art. Examples of such regulatory sequences include: a transcriptional promoter and enhancer or  
5 RNA polymerase binding sequence, a ribosomal binding sequence, including a translation initiation signal. Additionally, depending on the host cell chosen and the vector employed, other sequences, such as an origin of replication, additional DNA restriction sites, enhancers, and sequences conferring inducibility of transcription may be incorporated into  
10 the expression vector. It will also be appreciated that the necessary regulatory sequences may be supplied by the native A and B chains and/or its flanking regions.

The recombinant expression vectors of the invention may also contain a selectable marker gene which facilitates the selection of host  
15 cells transformed or transfected with a recombinant molecule of the invention. Examples of selectable marker genes are genes encoding a protein such as G418 and hygromycin which confer resistance to certain drugs,  $\beta$ -galactosidase, chloramphenicol acetyltransferase, firefly luciferase, or an immunoglobulin or portion thereof such as the Fc portion of an  
20 immunoglobulin preferably IgG. Transcription of the selectable marker gene is monitored by changes in the concentration of the selectable marker protein such as  $\beta$ -galactosidase, chloramphenicol acetyltransferase, or firefly luciferase. If the selectable marker gene encodes a protein conferring antibiotic resistance such as neomycin resistance transformant  
25 cells can be selected with G418. Cells that have incorporated the selectable marker gene will survive, while the other cells die. This makes it possible to visualize and assay for expression of recombinant expression vectors of the invention and in particular to determine the effect of a mutation on expression and phenotype. It will be appreciated that selectable markers  
30 can be introduced on a separate vector from the nucleic acid of interest.

The recombinant expression vectors may also contain genes which encode a fusion moiety which provides increased expression of the recombinant protein; increased solubility of the recombinant protein; and aid in the purification of the target recombinant protein by acting as a

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ligand in affinity purification. For example, a proteolytic cleavage site may be added to the target recombinant protein to allow separation of the recombinant protein from the fusion moiety subsequent to purification of the fusion protein. Typical fusion expression vectors include pGEX (Amrad  
5 Corp., Melbourne, Australia), pMAL (New England Biolabs, Beverly, MA) and pRIT5 (Pharmacia, Piscataway, NJ) which fuse glutathione S-transferase (GST), maltose E binding protein, or protein A, respectively, to the recombinant protein.

Recombinant expression vectors can be introduced into host  
10 cells to produce a transformant host cell. The term "transformant host cell" is intended to include prokaryotic and eukaryotic cells which have been transformed or transfected with a recombinant expression vector of the invention. The terms "transformed with", "transfected with", "transformation" and "transfection" are intended to encompass  
15 introduction of nucleic acid (e.g. a vector) into a cell by one of many possible techniques known in the art. Prokaryotic cells can be transformed with nucleic acid by, for example, electroporation or calcium-chloride mediated transformation. Nucleic acid can be introduced into mammalian cells via conventional techniques such as calcium phosphate or calcium  
20 chloride co-precipitation, DEAE-dextran mediated transfection, lipofectin, electroporation or microinjection. Suitable methods for transforming and transfecting host cells can be found in Sambrook et al. (Molecular Cloning: A Laboratory Manual, 2nd Edition, Cold Spring Harbor Laboratory press (1989)), and other laboratory textbooks.

25 Suitable host cells include a wide variety of prokaryotic and eukaryotic host cells. For example, the proteins of the invention may be expressed in bacterial cells such as *E. coli*, insect cells (using baculovirus), yeast cells or mammalian cells. Other suitable host cells can be found in Goeddel, Gene Expression Technology: Methods in Enzymology 185,  
30 Academic Press, San Diego, CA (1991).

More particularly, bacterial host cells suitable for carrying out the present invention include *E. coli*, *B. subtilis*, *Salmonella typhimurium*, and various species within the genus' *Pseudomonas*, *Streptomyces*, and *Staphylococcus*, as well as many other bacterial species well known to one

of ordinary skill in the art. Suitable bacterial expression vectors preferably comprise a promoter which functions in the host cell, one or more selectable phenotypic markers, and a bacterial origin of replication. Representative promoters include the  $\beta$ -lactamase (penicillinase) and

5 lactose promoter system (see Chang et al., *Nature* 275:615 (1978)), the trp promoter (Nichols and Yanofsky, *Meth in Enzymology* 101:155, (1983) and the tac promoter (Russell et al., *Gene* 20: 231, (1982)). Representative selectable markers include various antibiotic resistance markers such as the kanamycin or ampicillin resistance genes. Suitable expression vectors

10 include but are not limited to bacteriophages such as lambda derivatives or plasmids such as pBR322 (Bolivar et al., *Gene* 2:9S, (1977)), the pUC plasmids pUC18, pUC19, pUC118, pUC119 (see Messing, *Meth in Enzymology* 101:20-77, 1983 and Vieira and Messing, *Gene* 19:259-268 (1982)), and pNH8A, pNH16a, pNH18a, and Bluescript M13 (Stratagene, La

15 Jolla, Calif.). Typical fusion expression vectors which may be used are discussed above, e.g. pGEX (Amrad Corp., Melbourne, Australia), pMAL (New England Biolabs, Beverly, MA) and pRIT5 (Pharmacia, Piscataway, NJ). Examples of inducible non-fusion expression vectors include pTrc (Amann et al., *Gene* 69:301-315 (1988)) and pET 11d (Studier et al., *Gene*

20 *Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, California, 60-89 (1990)).

Yeast and fungi host cells suitable for carrying out the present invention include, but are not limited to *Saccharomyces cerevisiae*, the genera *Pichia* or *Kluyveromyces* and various species of the genus *Aspergillus*.

25 Examples of vectors for expression in yeast *S. cerevisiae* include pYepSec1 (Baldari. et al., *Embo J.* 6:229-234 (1987)), pMFa (Kurjan and Herskowitz, *Cell* 30:933-943 (1982)), pJRY88 (Schultz et al., *Gene* 54:113-123 (1987)), and pYES2 (Invitrogen Corporation, San Diego, CA). Protocols for the transformation of yeast and fungi are well known to those of ordinary skill

30 in the art.(see Hinnen et al., *Proc. Natl. Acad. Sci. USA* 75:1929 (1978); Itoh et al., *J. Bacteriology* 153:163 (1983), and Cullen et al. (*Bio/Technology* 5:369 (1987)).

Mammalian cells suitable for carrying out the present invention include, among others: COS (e.g., ATCC No. CRL 1650 or 1651),

BHK (e.g. ATCC No. CRL 6281), CHO (ATCC No. CCL 61), HeLa (e.g., ATCC No. CCL 2), 293 (ATCC No. 1573) and NS-1 cells. Suitable expression vectors for directing expression in mammalian cells generally include a promoter (e.g., derived from viral material such as polyoma, Adenovirus 2, cytomegalovirus and Simian Virus 40), as well as other transcriptional and translational control sequences. Examples of mammalian expression vectors include pCDM8 (Seed, B., *Nature* 329:840 (1987)) and pMT2PC (Kaufman et al., *EMBO J.* 6:187-195 (1987)).

Given the teachings provided herein, promoters, terminators, and methods for introducing expression vectors of an appropriate type into plant, avian, and insect cells may also be readily accomplished. For example, within one embodiment, the proteins of the invention may be expressed from plant cells (see Sinkar et al., *J. Biosci* (Bangalore) 11:47-58 (1987), which reviews the use of *Agrobacterium* rhizogenes vectors; see also Zambryski et al., *Genetic Engineering, Principles and Methods*, Hollaender and Setlow (eds.), Vol. VI, pp. 253-278, Plenum Press, New York (1984), which describes the use of expression vectors for plant cells, including, among others, pAS2022, pAS2023, and pAS2034).

Insect cells suitable for carrying out the present invention include cells and cell lines from *Bombyx*, *Trichoplusia* or *Spodoptera* species. Baculovirus vectors available for expression of proteins in cultured insect cells (SF 9 cells) include the pAc series (Smith et al., *Mol. Cell Biol.* 3:2156-2165 (1983)) and the pVL series (Lucklow, V.A., and Summers, M.D., *Virology* 170:31-39 (1989)). Some baculovirus-insect cell expression systems suitable for expression of the recombinant proteins of the invention are described in PCT/US/02442.

Alternatively, the proteins of the invention may also be expressed in non-human transgenic animals such as, rats, rabbits, sheep and pigs (Hammer et al. *Nature* 315:680-683 (1985); Palmiter et al. *Science* 222:809-814 (1983); Brinster et al. *Proc. Natl. Acad. Sci. USA* 82:4438-4442 (1985); Palmiter and Brinster *Cell* 41:343-345 (1985) and U.S. Patent No. 4,736,866).



The proteins of the invention may also be prepared by chemical synthesis using techniques well known in the chemistry of proteins such as solid phase synthesis (Merrifield, *J. Am. Chem. Assoc.* 85:2149-2154 (1964)) or synthesis in homogenous solution (Houbenweyl, 5 *Methods of Organic Chemistry*, ed. E. Wansch, Vol. 15 I and II, Thieme, Stuttgart (1987)).

The present invention also provides proteins comprising an A chain of a ricin-like toxin, a B chain of a ricin-like toxin and a heterologous linker amino acid sequence linking the A and B chains, 10 wherein the linker sequence contains a cleavage recognition site for a disease-specific protease. Such a protein could be prepared other than by recombinant means, for example by chemical synthesis or by conjugation of A and B chains and a linker sequence isolated and purified from their natural plant, fungal or bacterial source. Such A and B chains could be 15 prepared having the glycosylation pattern of the native ricin-like toxin.

N-terminal or C-terminal fusion proteins comprising the protein of the invention conjugated with other molecules, such as proteins may be prepared by fusing, through recombinant techniques. The resultant fusion proteins contain a protein of the invention fused to the 20 selected protein or marker protein as described herein. The recombinant protein of the invention may also be conjugated to other proteins by known techniques. For example, the proteins may be coupled using heterobifunctional thiol-containing linkers as described in WO 90/10457, N-succinimidyl-3-(2-pyridyldithio-propionate) or N-succinimidyl-5 25 thioacetate. Examples of proteins which may be used to prepare fusion proteins or conjugates include cell binding proteins such as immunoglobulins, hormones, growth factors, lectins, insulin, low density lipoprotein, glucagon, endorphins, transferrin, bombesin, asialoglycoprotein glutathione-S-transferase (GST), hemagglutinin (HA), 30 and truncated myc.

#### **Utility of the Nucleic Acid Molecules and Proteins of the Invention**

The proteins of the invention may be used to specifically inhibit or destroy mammalian cells affected by a disease or infection which have associated with such cells a specific protease, i.e., disease-specific, for

example cancer cells or cells infected with a virus, fungus or parasite, all of which are encompassed within the term "disease-specific." It is an advantage of the recombinant proteins of the invention that they have specificity for said cells without the need for a cell binding component. The  
5 ricin-like B chain of the recombinant proteins recognize galactose moieties on the cell surface and ensure that the protein is taken up by the diseased cell and released into the cytoplasm. When the protein is internalized into a non-infected cell, cleavage of the heterologous linker would not occur in the absence of the disease-specific protease and the A chain will remain  
10 inactive bound to the B chain. Conversely, when the protein is internalized into a diseased cell, the disease-specific protease will cleave the cleavage recognition site in the linker thereby releasing the toxic A chain.

The specificity of a recombinant protein of the invention may be tested by treating the protein with the disease-specific protease  
15 which is thought to be specific for the cleavage recognition site of the linker and assaying for cleavage products. Disease-specific proteases may be isolated from cancer cells or infected cells, or they may be prepared recombinantly, for example following the procedures in Darket et al. (*J. Biol. Chem.* 254:2307-2312 (1988)). The cleavage products may be identified  
20 for example based on size, antigenicity or activity. The toxicity of the recombinant protein may be investigated by subjecting the cleavage products to an *in vitro* translation assay in cell lysates, for example using Brome Mosaic Virus mRNA as a template. Toxicity of the cleavage products may be determined using a ribosomal inactivation assay (Westby  
25 et al., *Bioconjugate Chem.* 3:377-382 (1992)). The effect of the cleavage products on protein synthesis may be measured in standardized assays of *in vitro* translation utilizing partially defined cell free systems composed for example of a reticulocyte lysate preparation as a source of ribosomes and various essential cofactors, such as mRNA template and amino acids. Use  
30 of radiolabelled amino acids in the mixture allows quantitation of incorporation of free amino acid precursors into trichloroacetic acid precipitable proteins. Rabbit reticulocyte lysates may be conveniently used (O'Hare, *FEBS Lett.* 273:200-204 (1990)).

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A further model may be used to test the cytotoxicity of recombinant proteins having a heterologous linker sequence containing a cleavage recognition site for a cancer-associated Cathepsin B protease is provided in human glioma (Mikkelsen, T. et al. *J. Neurosurg*, 83:285-290 (1995)).

Similarly, the cytotoxicity of recombinant proteins having a heterologous linker sequence containing a cleavage recognition site for a malarial protease may be tested by a Plasmodium invasion assay using human erythrocytes infected with mature-stage merozoite parasites as described by McPherson, R.A. et al. (*Mol. Biochem. Parasitol.* 62:233-242 (1993)). Alternatively, in vitro cultures of human hepatic parenchymal cells may be used to evaluate schizont infectivity and Plasmodium merozoite generation.

With respect to models of viral infection and replication, suitable animal cells which can be cultured *in vitro* and which are capable of maintaining viral replication can be used as hosts. The toxicity of the recombinant protein for infected and non-infected cultures may then be compared. The ability of the recombinant protein of the invention to inhibit the expression of these viral antigens may be an important indicator of the ability of the protein to inhibit viral replication. Levels of these antigens may be measured in assays using labelled antibodies having specificity for the antigens. Inhibition of viral antigen expression has been correlated with inhibition of viral replication (U.S. Patent No. 4,869,903). Toxicity may also be assessed based on a decrease in protein synthesis in target cells, which may be measured by known techniques, such as incorporation of labelled amino acids, such as [3H] leucine (O'Hare et al., *FEBS Lett.* 273:200-204 (1990)). Infected cells may also be pulsed with radiolabelled thymidine and incorporation of the radioactive label into cellular DNA may be taken as a measure of cellular proliferation. Toxicity may also be measured based on cell death or lysis, for example, the viability of infected and non-infected cell cultures exposed to the recombinant protein may be compared. Cell viability may be assessed by known techniques, such as trypan blue exclusion assays.

Although the primary specificity of the proteins of the invention for diseased cells is mediated by the specific cleavage of the cleavage recognition site of the linker, it will be appreciated that specific cell binding components may optionally be conjugated to the proteins of the invention. Such cell binding components may be expressed as fusion proteins with the proteins of the invention or the cell binding component may be physically or chemically coupled to the protein component. Examples of suitable cell binding components include antibodies to cancer, viral or parasitic proteins.

Antibodies having specificity for a cell surface protein may be prepared by conventional methods. A mammal, (e.g. a mouse, hamster, or rabbit) can be immunized with an immunogenic form of the peptide which elicits an antibody response in the mammal. Techniques for conferring immunogenicity on a peptide include conjugation to carriers or other techniques well known in the art. For example, the peptide can be administered in the presence of adjuvant. The progress of immunization can be monitored by detection of antibody titers in plasma or serum. Standard ELISA or other immunoassay procedures can be used with the immunogen as antigen to assess the levels of antibodies. Following immunization, antisera can be obtained and, if desired, polyclonal antibodies isolated from the sera.

To produce monoclonal antibodies, antibody producing cells (lymphocytes) can be harvested from an immunized animal and fused with myeloma cells by standard somatic cell fusion procedures thus immortalizing these cells and yielding hybridoma cells. Such techniques are well known in the art, (e.g. the hybridoma technique originally developed by Kohler and Milstein (*Nature* 256:495-497 (1975)) as well as other techniques such as the human B-cell hybridoma technique (Kozbor et al., *Immunol. Today* 4:72 (1983)), the EBV-hybridoma technique to produce human monoclonal antibodies (Cole et al., *Monoclonal Antibodies in Cancer Therapy* Allen R., Bliss, Inc., pages 77-96 (1985)), and screening of combinatorial antibody libraries (Huse et al., *Science* 246:1275 (1989)). Hybridoma cells can be screened immunochemically for production of

antibodies specifically reactive with the peptide and the monoclonal antibodies can be isolated.

The term "antibody" as used herein is intended to include fragments thereof which also specifically react with a cell surface component. Antibodies can be fragmented using conventional techniques and the fragments screened for utility in the same manner as described above. For example, F(ab')<sub>2</sub> fragments can be generated by treating antibody with pepsin. The resulting F(ab')<sub>2</sub> fragment can be treated to reduce disulfide bridges to produce Fab' fragments.

Chimeric antibody derivatives, i.e., antibody molecules that combine a non-human animal variable region and a human constant region are also contemplated within the scope of the invention. Chimeric antibody molecules can include, for example, the antigen binding domain from an antibody of a mouse, rat, or other species, with human constant regions. Conventional methods may be used to make chimeric antibodies containing the immunoglobulin variable region which recognizes a cell surface antigen (See, for example, Morrison et al., *Proc. Natl Acad. Sci. U.S.A.* 81:6851 (1985); Takeda et al., *Nature* 314:452 (1985), Cabilly et al., U.S. Patent No. 4,816,567; Boss et al., U.S. Patent No. 4,816,397; Tanaguchi et al., E.P. Patent No. 171,496; European Patent No. 173,494, United Kingdom Patent No. GB 2177096B). It is expected that chimeric antibodies would be less immunogenic in a human subject than the corresponding non-chimeric antibody.

Monoclonal or chimeric antibodies specifically reactive against cell surface components can be further humanized by producing human constant region chimeras, in which parts of the variable regions, particularly the conserved framework regions of the antigen-binding domain, are of human origin and only the hypervariable regions are of non-human origin. Such immunoglobulin molecules may be made by techniques known in the art, (e.g. Teng et al., *Proc. Natl. Acad. Sci. U.S.A.*, 80:7308-7312 (1983); Kozbor et al., *Immunology Today* 4:7279 (1983); Olsson et al., *Meth. Enzymol.*, 92:3-16 (1982), and PCT Publication WO92/06193 or EP 239,400). Humanized antibodies can also be commercially produced (Scotgen Limited, 2 Holly Road, Twickenham, Middlesex, Great Britain.)

Specific antibodies, or antibody fragments, reactive against cell surface components may also be generated by screening expression libraries encoding immunoglobulin genes, or portions thereof, expressed in bacteria with cell surface components. For example, complete Fab  
5 fragments, VH regions and FV regions can be expressed in bacteria using phage expression libraries (See for example Ward et al., *Nature* 341:544-546 (1989); Huse et al., *Science* 246:1275-1281 (1989); and McCafferty et al., *Nature* 348:552-554 (1990)). Alternatively, a SCID-hu mouse, for example the model developed by Genpharm, can be used to produce antibodies, or  
10 fragments thereof.

The proteins of the invention may be formulated into pharmaceutical compositions for administration to subjects in a biologically compatible form suitable for administration *in vivo*. By "biologically compatible form suitable for administration in vivo" is meant a form of the  
15 substance to be administered in which any toxic effects are outweighed by the therapeutic effects. The substances may be administered to living organisms including humans, and animals. Administration of a therapeutically active amount of the pharmaceutical compositions of the present invention is defined as an amount effective, at dosages and for  
20 periods of time necessary to achieve the desired result. For example, a therapeutically active amount of a substance may vary according to factors such as the disease state, age, sex, and weight of the individual, and the ability of antibody to elicit a desired response in the individual. Dosage regime may be adjusted to provide the optimum therapeutic response.  
25 For example, several divided doses may be administered daily or the dose may be proportionally reduced as indicated by the exigencies of the therapeutic situation.

The nucleic acid molecules of the invention may be formulated into pharmaceutical compositions for administration to subjects  
30 in a biologically compatible form suitable for administration *in vivo*. By "biologically compatible form suitable for administration in vivo" is meant a form of the substance to be administered in which any toxic effects are outweighed by the therapeutic effects. The substances may be administered to living organisms including humans, and animals.

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Administration of a therapeutically active amount of the pharmaceutical compositions of the present invention is defined as an amount effective, at dosages and for periods of time necessary to achieve the desired result. For example, a therapeutically active amount of a substance may vary  
5 according to factors such as the disease state, age, sex, and weight of the individual, and the ability of antibody to elicit a desired response in the individual. Dosage regime may be adjusted to provide the optimum therapeutic response. For example, several divided doses may be administered daily or the dose may be proportionally reduced as indicated  
10 by the exigencies of the therapeutic situation.

The active substance may be administered in a convenient manner such as by injection (subcutaneous, intravenous, intramuscular, etc.), oral administration, inhalation, transdermal administration (such as topical cream or ointment, etc.), or suppository applications. Depending  
15 on the route of administration, the active substance may be coated in a material to protect the compound from the action of enzymes, acids and other natural conditions which may inactivate the compound.

The compositions described herein can be prepared by *per se* known methods for the preparation of pharmaceutically acceptable  
20 compositions which can be administered to subjects, such that an effective quantity of the active substance is combined in a mixture with a pharmaceutically acceptable vehicle. Suitable vehicles are described, for example, in Remington's Pharmaceutical Sciences (Remington's Pharmaceutical Sciences, Mack Publishing Company, Easton, Pa., USA  
25 1985). On this basis, the compositions include, albeit not exclusively, solutions of the substances in association with one or more pharmaceutically acceptable vehicles or diluents, and contained in buffered solutions with a suitable pH and iso-osmotic with the physiological fluids.

The pharmaceutical compositions may be used in methods  
30 for treating animals, including mammals, preferably humans, with cancer or infected with a virus or a parasite. It is anticipated that the compositions will be particularly useful for treating patients with B-cell lymphoproliferative disease, (melanoma), mononucleosis, cytomegalic inclusion disease, malaria, herpes, shingles, hepatitis, poliomyelitis, or



infectious laryngotracheitis. The dosage and type of recombinant protein to be administered will depend on a variety of factors which may be readily monitored in human subjects. Such factors include the etiology and severity (grade and stage) of neoplasia, the stage of malarial infection (e.g. 5 exoerythrocytic *vs.* erythrocytic), or antigen levels associated with viral load in patient tissues or circulation.

As mentioned above, the novel recombinant toxic proteins and nucleic acid molecules of the present invention are useful in treating cancerous or infected cells wherein the cells contain a specific protease that 10 can cleave the linker region of the recombinant toxic protein. One skilled in the art can appreciate that many different recombinant toxic proteins can be prepared once a disease associated protease has been identified. For example, the novel recombinant toxic proteins and nucleic acid molecules of the invention may be used to treat CNS tumors. Muller et al. 15 (1993) describe increased activity of Insulin-type Growth Factor Binding Protein-3 (IGFBP-3) protease in the Cerebral Spinal Fluid of patients with CNS tumors. Cohen et al. (1992) claim that prostate-specific antigen (PSA) is an IGFBP-3 protease. The pAP290 construct described above is a substrate for PSA. Conover et al. (1994) claim that cathepsin D is IGFBP-3 20 protease. The pAP276 described herein is a substrate for cathepsin D. Another example of a specific use of the invention is treatment of human glioma which has been shown to produce cathepsin D (Mikkelsen, T. et al. *J. Neurosurg.*, 83:285-290 (1995)). The pAP 214 and 272 define herein are substrates for cathepsin B.

25 In addition, the novel proteins and nucleic acid molecules of the present invention may be used to treat cystic fibrosis. Hansen et al. (1995) describe how CF airway disease is characterized by neutrophil-dominated chronic inflammation with an excess of uninhibited neutrophil elastase (NE). NE levels in CF sputum are 350 times higher than that 30 found in normal sputum. The pAP294 described herein is a substrate for neutrophil elastase.

As well, the novel proteins and nucleic acid molecules of the present invention may also be used to treat multiple sclerosis. Bever Jr. et al. (1994) implicate cathepsin B (possibly from inflammatory cells of

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hematogenous origin) in the demyelination found in multiple sclerosis. pAPs 214 and 272 defined herein present substrates for cathepsin B.

The term "animal" as used herein includes all members of the animal kingdom including mammals, preferably humans.

5                   The following non-limiting examples are illustrative of the present invention:

### **EXAMPLES**

#### **Example 1**

#### **Cloning and Expression of Proricin Variants Activated by Disease-Specific Proteases**

10                   Isolation of total RNA

#### **Isolation of total RNA**

                  The preproricin gene was cloned from new foliage of the castor bean plant. Total messenger RNA was isolated according to established procedures (Sambrook et al., *Molecular Cloning: A Lab Manual* (Cold Spring Harbour Press, Cold Spring Harbour, (1989)) and cDNA  
15                   generated using reverse transcriptase.

#### **cDNA Synthesis:**

                  Oligonucleotides, corresponding to the extreme 5' and 3' ends of the preproricin gene were synthesized and used to PCR amplify  
20                   the gene. Using the cDNA sequence for preproricin (Lamb et al., Eur. J. Biochem., 145:266-270, 1985), several oligonucleotide primers were designed to flank the start and stop codons of the preproricin open reading frame. The oligonucleotides were synthesized using an Applied Biosystems Model 392 DNA/RNA Synthesizer. First strand cDNA  
25                   synthesis was primed using the oligonucleotide Ricin1729C (Table 1). Three micrograms of total RNA was used as a template for oligo Ricin1729C primed synthesis of cDNA using Superscript II Reverse Transcriptase (BRL) following the manufacturer's protocol.

#### **DNA Amplification and Cloning**

30                   The first strand cDNA synthesis reaction was used as template for DNA amplification by the polymerase chain reaction (PCR). The preproricin cDNA was amplified using the upstream primer Ricin-99 and the downstream primer Ricin1729C with Vent DNA polymerase (New England Biolabs) using standard procedures (Sambrook et al., *Molecular*

Cloning: A Laboratory Manual, Second Edition, (Cold Spring Harbor Laboratory Press, 1989)). Amplification was carried out in a Biometra thermal cycler (TRIO-Thermalcycler) using the following cycling parameters: denaturation 95°C for 1 min., annealing 52°C for 1 min., and  
5 extension 72°C for 2 min., (33 cycles), followed by a final extension cycle at 72°C for 10 min. The 1846bp amplified product was fractionated on an agarose gel (Sambrook et al., Molecular Cloning: A Laboratory Manual, Second Edition, (Cold Spring Harbor Laboratory Press, 1989), and the DNA purified from the gel slice using Qiaex resin (Qiagen) following the  
10 manufacturer's protocol. The purified PCR fragment encoding the preproricin cDNA was then ligated (Sambrook et al., Molecular Cloning: A Laboratory Manual, Second Edition, (Cold Spring Harbor Laboratory Press, 1989)) into an Eco RV-digested pBluescript II SK plasmid (Stratagene), and used to transform competent XL1-Blue cells (Stratagene).  
15 Positive clones were confirmed by restriction digestion of purified plasmid DNA. Plasmid DNA was extracted using a Qiaprep Spin Plasmid Miniprep Kit (Qiagen).

#### DNA Sequencing

The cloned PCR product containing the putative preproricin  
20 gene was confirmed by DNA sequencing of the entire cDNA clone (pAP-144). Sequencing was performed using an Applied Biosystems 373A Automated DNA Sequencer, and confirmed by double-stranded dideoxy sequencing by the Sanger method using the Sequenase kit (USB). The oligonucleotide primers used for sequencing were as follows: Ricin267,  
25 Ricin486, Ricin725, Ricin937, Ricin1151, Ricini1399, Ricin1627, T3 primer (5'AATTAACCCTCACTAAAGGG-3') (SEQ ID NO. 128) and T7 primer (5'GTAATACGACTCACTATAGGGC-3) (SEQ ID NO. 129). Sequence data was compiled and analyzed using PC Gene software package (intelligenetics). The sequences and location of oligonucleotide primers is  
30 shown in Table 1. The oligonucleotide primers shown in Table 1 have been assigned the following sequence ID numbers:  
Ricin-109 is referred to herein as SEQ ID NO. 130;  
Ricin-99Eco is referred to herein as SEQ ID NO. 131;  
Ricin267 is referred to herein as SEQ ID NO. 132;

Ricin486 is referred to herein as SEQ ID NO. 133;

Ricin725 is referred to herein as SEQ ID NO. 134;

Ricin 937 is referred to herein as SEQ ID NO. 135;

Ricin 1151 is referred to herein as SEQ ID NO. 136;

5 Ricin 1399 is referred to herein as SEQ ID NO. 137;

Ricin 1627 is referred to herein as SEQ ID NO. 138;

Ricin 1729C is referred to herein as SEQ ID NO. 139; and

Ricin 1729C Xba is referred to herein as SEQ ID NO. 140.

#### Production and Cloning of Linker Variants

10 pAP144 cut with EcoRI was used as target for PCR pairs  
employing the Ricin109-Eco oligonucleotide (Ricin-109Eco primer: 5-  
GGAGGAATCCGGAGATGAAACCGGGAGGAAATACTATTGTAAT-3  
(SEQ ID No. 141)) and a mutagenic primer for the 5' half of the linker as  
well as the Ricin1729PstI primer (Ricin1729-PstI: 5-  
15 GTAGGCGCTGCAGATAACTTGCTGTCCTTTCAG-3 (SEQ ID No. 142))  
and a mutagenic primer for the 3' half of the linker. The cycling conditions  
used for the PCRs were 98 degrees C for 2 min.; 98C 1 min., 52C 1 min.,  
72C 1 min. 15 sec. (30 cycles); 72 degrees C 10min.; 4 degrees C soak. The  
PCR products were then digested by EcoRI and PstI respectively,  
20 electrophoresed on an agarose gel, and the bands purified by via glass  
wool spin columns. Triple ligations comprising the PCR product pairs  
(corresponding halves of the new linker) and pVL1393 vector digested  
with EcoRI and PstI were carried out. Recombinant clones were identified  
by restriction digests of plasmid miniprep DNA and the altered linkers  
25 confirmed by DNA sequencing. See Figure 45 as an example of the cloning  
strategy. Recombinant clones were identified by restriction digests of  
plasmid miniprep DNA and the altered linkers confirmed by DNA  
sequencing. Note that since all altered linker variants were cloned directly  
into the pVL1393 vector odd-numbered pAPs were no longer required or  
30 produced.

#### Isolation of Recombinant Baculoviruses

Insect cells *S. frugiperda* (Sf9), and *Trichoplusia ni* (Tn368 and  
BTI-TN-581-4 (High Five)) were maintained on EX-CELL 405 medium  
(JRH Biosciences) supplemented with 10% total calf serum (Summers et al.,

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A Manual of Methods of Baculovirus Vectors and Insect Cell Culture Procedures, (Texas Agricultural Experiment Station, 1987)). Two micrograms of recombinant pVL1393 DNA was co-transfected with 0.5 microgram of BaculoGold AcNPV DNA (Pharmingen) into  $2 \times 10^6$  Tn368 insect cells following the manufacturer's protocol (Gruenwald et al., Baculovirus Expression Vector System: Procedures and Methods Manual, 2nd Edition, (San Diego, CA, 1993)). On day 5 post-transfection, media were centrifuged and the supernatants tested in limiting dilution assays with Tn368 cells (Summers et al., A Manual of Methods of Baculovirus Vectors and Insect Cell Culture Procedures, (Texas Agricultural Experiment Station, 1987)). Recombinant viruses in the supernatants were then amplified by infecting Tn368 cells at a multiplicity of infection (moi) of 0.1, followed by collection of day 3 to 5 supernatants. A total of three rounds of amplification were performed for each recombinant following established procedures (Summers et al., A Manual of Methods of Baculovirus Vectors and Insect Cell Culture Procedures, (Texas Agricultural Experiment Station, 1987 and Gruenwald et al., Baculovirus Expression Vector System: Procedures and Methods Manual, 2nd Edition, (San Diego, CA, 1993)).

#### 20 Expression of Mutant Proricin

Recombinant baculoviruses were used to infect  $1 \times 10^7$  Tn368 or sf9 cells at an moi of 9 in EX-CELL 405 media (JRH Biosciences) with 25mM  $\alpha$ -lactose in spinner flasks. Media supernatants containing mutant proricens were collected 3 or 4 days post-infection.

#### 25 EXAMPLE 2

##### Harvesting and affinity column purification of pro-ricin variants

Protein samples were harvested three days post transfection. The cells were removed by centrifuging the media at 8288 g for ten minutes using a GS3 (Sorvall) centrifuge rotor. The supernatant was further clarified by centrifuging at 25400 g using a SLA-1500 rotor (Sorvall) for 45 minutes. Protease inhibitor phenylmethanesulfonyl fluoride (Sigma) was slowly added to a final concentration of 1mM. The samples were further prepared by adding lactose to a concentration of 20 mM (not including the previous lactose contained in the expression medium). The

samples were concentrated to 700 mL using a Prep/Scale-TFF Cartridge (2.5ft, 10K regenerated cellulose (Millipore)) and a Masterflex pump. The samples were then dialysed for 2 days in 1X Column Buffer (50 mM Tris, 100 mM NaCl, 0.02% NaN<sub>3</sub>, pH 7.5) using dialysis tubing (10 K MWCO, 32 mm flat width(Spectra/Por)). Subsequently, the samples were clarified by centri fusing at 25400 g using a SLA-1500 rotor (Sorvall) for 45 minutes.

Following centrifugation, the samples were degassed and applied at 4 degrees C to a XK26/20 (Pharmacia) column (attached to a Pharmacia peristaltic pump, Pharmacia Single-path Monitor UV-1 Control and Optical Units, and Bromma LKB 2210 2-Channel Recorder) containing 20 mL of a-Lactose Agarose Resin (Sigma). The column was washed for 3 hours with 1X Column buffer. Elution of pro-ricin variant was performed by eluting with buffer (1X Column buffer (0.1% NaN<sub>3</sub>), 100 mM Lactose) until the baseline was again restored. The samples were concentrated using an Amicon 8050 concentrator (Amicon) with a YM10 76 mm membrane, utilizing argon gas to pressurize the chamber. The samples were further concentrated in Centricon 10 (Millipore) concentrators according to manufacturer's specifications.

#### **Purification of Variant pAP-Protein by gel filtration chromatography**

In order to purify the pro-ricin variant from processed material produced during fermentation, the protein was applied to a SUPERDEX 75 (16/60) column and SUPERDEX 200 (16/60) column (Pharmacia) connected in series equilibrated with 50 mM Tris, 100mM NaCl, pH 7.5 containing 100 mM Lactose and 0.1%  $\beta$ -mercaptoethanol ( $\beta$ ME). The flow rate of the column was 0.15 mL/min and fractions were collected every 25 minutes. The UV (280 nm) trace was used to determine the approximate location of the purified pAP-protein and thus determine the samples for Western analysis.

#### **Western analysis of column fractions**

Fractions eluted from the SUPERDEX columns (Pharmacia) were analyzed for purity using standard Western blotting techniques. An aliquot of 10 $\mu$ L from each fraction was boiled in 1X sample buffer (62.6 mM Tris-C1, pH 6.8, 4.4%  $\beta$ ME, 2% sodium dodecyl sulfate (SDS), 5% glycerol (all from Sigma) and 0.002% bromophenol blue (Biorad)) for five

minutes. Denatured samples were loaded on 12% Tris-Glycine Gels (Biorad) along with 50 ng of RCA<sub>60</sub> (Sigma) and 5 µL of kaleidoscope prestained standards (Biorad). Electrophoresis was carried out for ninety minutes at 100V in 25 mM Tris-Cl, pH 8.3, 0.1% SDS, and 192 mM glycine using the BioRad Mini Protean II cells (Biorad).

Following electrophoresis gels were equilibrated in transfer buffer (48 mM Tris, 39 mM glycine, 0.0375% SDS, and 20% Methanol) for a few minutes. PVDF Biorad membrane was presoaked for one minute in 100% methanol, rinsed in ddH<sub>2</sub>O and two minutes in transfer buffer. Whatman paper was soaked briefly in transfer buffer. Five pieces of Whatman paper, membrane, gel, and another five pieces of Whatman paper were arranged on the bottom cathode (anode) of the Pharmacia Novablot transfer apparatus (Pharmacia). Transfer was for one hour at constant current (2 mA/cm<sup>2</sup>).

Transfer was confirmed by checking for the appearance of the prestained standards on the membrane. Non-specific sites on the membrane were blocked by incubating the blot for thirty minutes in 1X Phosphate Buffered Saline (1X PBS; 137 mM NaCl, 2.7 mM KCl, 8 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.5 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.4) with 5% skim milk powder (Carnation). Primary antibody (Rabbit α-ricin, Sigma) was diluted 1:3000 in 1X PBS containing 0.1% Tween 20 (Sigma) and 2.5% skim milk and incubated with blot for forty five minutes on a orbital shaker (VWR). Non-specifically bound primary antibody was removed by washing the blot for ten minutes with 1X PBS containing 0.2% Tween 20. This was repeated four times. Secondary antibody donkey anti-rabbit (Amersham) was incubated with the blot under the same conditions as the primary antibody. Excess secondary antibody was washed as described above. Blots were developed with the ECL Western Blotting detection reagents according to the manufacturer's instructions. Blots were exposed to Medtec's Full Speed Blue Film (Medtee) or Amersham's ECL Hyperfilm (Amersham) for one second to five minutes. Film was developed in a KODAK Automatic Developer.

**Determination of lectin binding ability of pro-ricin variant**

An Immulon 2 plate (VDVR) was coated with 100 µl per well of 10µg/ml of asialofetuin and left overnight at 4°C. The plate was washed with 3X 300 µL per well with ddH<sub>2</sub>O using an automated plate washer (BioRad). The plate was blocked for one hour at 37°C by adding 300 µL per well of PBS containing 1% ovalbumin. The plate was washed again as above. Pro-ricin variant pAP-protein was added to the plate in various dilutions in 1X Baculo. A standard curve of RCA<sub>60</sub> (Sigma) from 1-10 ng was also included. The plate was incubated for 1 h at 37°C. The plate was washed as above. Anti-ricin monoclonal antibody (Sigma) was diluted 1:3000 in 1X PBS containing 0.5% ovalbumin and 0.1% tween-20, added at 100 µL per well and incubated for 1 h at 37°C. The plate was washed as above. Donkey-anti rabbit polyclonal antibody was diluted 1:3000 in 1X PBS containing 0.5% ovalbumin, 0.1% Tween-20, and added at 100µL per well and incubated for 1 h at 37°C. The plate was given a final wash as described above. Substrate was added to plate at 100µL per well (1 mg/ml o-phenylenediamine (Sigma), 1 µL/ml H<sub>2</sub>O<sub>2</sub>, 25 µL of stop solution (20% H<sub>2</sub>SO<sub>4</sub>) was added and the absorbance read (A490nm-A630nm) using a SPECTRA MAX 340 plate reader (Molecular Devices).

**Determination of pAP -Protein activity using the rabbit reticulocyte assay**

- 20 Ricin samples were prepared for reduction.
- A) RCA<sub>60</sub> = 3,500 ng/µL of RCA<sub>60</sub> + 997 µL 1xEndo buffer  
(25mM Tris, 25mM KCl, 5mM MgCl<sub>2</sub>, pH 7.6)  
Reduction = 95 µL of 10ng/µL + 5 µL β-mercaptoethanol
- B) Ricin variants
- 25 Reduction = 40 µL variant + 2 µL β-mercaptoethanol  
The ricin standard and the variants were incubated for 30 minutes at room temperature.

**Ricin - Rabbit Reticulocyte lysate reaction**

- The required number of 0.5 mL tubes were labelled. (2  
30 tubes for each sample, + and - aniline). To each of the sample tubes 20 µL of 1X endo buffer was added, and 30 µL of buffer was added to the controls. To the sample tubes either 10 µL of 10ng/µL Ricin or 10µL of



variant was added. Finally, 30µL of rabbit reticulocyte lysate was added to all the tubes. The samples were incubated for 30 minutes at 30°C using the thermal block. Samples were removed from the eppendorf tube and contents added into a 1.5 mL tube containing 1 mL of TRIZOL (Gibco).

5 Samples were incubated for 15 minutes at room temperature. After the incubation, 200 µL of chloroform was added, and the sample was vortexed and spun at 12,000 g for 15 minutes at 4°C. The top aqueous layer from the samples was removed and contents added to a 1 mL tube containing 500 µL of isopropanol. Samples were incubated for 15 minutes at room

10 temperature and then centrifuged at 12,000 for 15 minutes at 4°C. Supernatant was removed and the pellets were washed with 1 mL of 70% ethanol. Centrifugation at 12,000 g for 5 minutes at 4°C precipitated the RNA. All but approximately 20 µL of the supernatant was removed and air dried. Pellets from the other samples (+aniline samples) were dissolved

15 in 20 µL of DEPC treated ddH<sub>2</sub>O. An 80 µL aliquot of 1 M aniline (distilled) with 2.8 M acetic acid was added to these RNA samples and transferred to a fresh 0.5 mL tube. The samples were incubated in the dark for 3 minutes at 60°C. RNA was precipitated by adding 100 µL of 95% ethanol and 5µL of 3M sodium acetate, pH 5.2 to each tube and centrifuging at 12,000 g for

20 30 minutes at 4°C. Pellets were washed with 1 mL 70% ethanol and centrifuged again at 12,000g for 5 minutes at 4°C to precipitate RNA. The supernatant was removed and air dried. These pellets were dissolved in 10µL of 0.1 X E buffer. To all samples, 10 µL of formamide loading dye was added. The RNA ladder (8 µL of ladder + 8 µL of loading dye) was

25 also included. Samples were incubated for 2 minutes at 70°C on the thermal block. Electrophoresis was carried out on the samples using 1.2% agarose, 50% formamide gels in 0.1X E buffer + 0.2% SDS. The gel was run for 90 minutes at 75 watts. RNA was visualized by staining the gel in 1 µg/µL ethidium bromide in running buffer for 45 minutes. The gel was

30 examined on a 302 nm UV box, photographed using the gel documentation system and saved to a computer disk.

### **Results:**

#### **Protein Expression Yields**

Aliquots were taken at each stop of the harvesting/purification and tested. Yields of functional ricin variant were determined by ELISA. Typical results of an 2400 mL prep of infected *T. ni* cells are given below.

5	<u>Aliquot</u>	<u>µg pAP 220</u>
	Before concentration and dialysis	6000
	After concentration and dialysis	4931
	alpha- Lactose agarose column flow through	219
10	alpha- Lactose agarose column elution	1058

Yield:  $1058/6000 = 17.6\%$

#### **Purification of pAP -Protein and Western Analysis of column fractions**

Partially purified pAP-protein was applied to Superdex 75 and 200 (16/60) columns connected in series in order to remove the contaminating non-specifically processed pAP-protein. Eluted fractions were tested via Western analysis as described above and the fractions containing the most pure protein were pooled, concentrated and re-applied to the column. The variant was applied a total of three times to the column. Final purified pAP-protein has less than 1% processed variant.

The purified pAP-protein was tested for susceptibility to cleavage by the particular protease and for activation of the A-chain of the pro-ricin variant, (inhibition of protein synthesis). Typically, pAP-protein was incubated with and without protease for a specified time period and then electrophoresed and blotted. Cleaved pAP will run as two 30 kDa proteins (B is slightly larger) under reducing (SDS-PAGE) conditions. Unprocessed pAP-protein, which contains the linker region, will run at 60 kDa.

#### **Activation of pAP -Protein variant with Specific Protease**

Activation of protease treated pAP-protein is based on the method of May *et al.* (EMBO Journal. 8 301-8, 1989). Activation of ricin A chain upon cleavage of the intermediary linker results in catalytic depurination of the adenosine 4325 residue of 28S or 26S rRNA. This depurination renders the molecule susceptible to amine-catalyzed hydrolysis by aniline of the

- phosphodiester bond on either side of the modification site. The result is a diagnostic 390 base band. As such, reticulocyte ribosomes incubated with biochemically purified ricin A chain, released the characteristic RNA fragment upon aniline treatment of isolated rRNA (May, M.J. et al. *Embo. Journal*, 8:301-308 at 302-303 (1989)). It is on this basis that the assay allows for the determination of activity of a ricin A chain which has been cleaved from the intact unit containing a particular variant linker sequence.

### **EXAMPLE 3**

#### **In Vitro Protease Digestion of Proricin Variants:**

- 10 Affinity-purified proricin variant is treated with individual disease-specific proteases to confirm specific cleavage in the linker region. Ricin-like toxin variants are eluted from the lactose-agarose matrix in protease digestion buffer (50mM NaCl, 50mM Na-acetate, pH 5.5, 1mM dithiothreitol) containing 100mM lactose. Proricin substrate is then
- 15 incubated at 37°C for 60 minutes with a disease-specific protease. The cleavage products consisting ricin A and B chains are identified using SDS/PAGE (Sambrook et al., *Molecular Cloning: a Laboratory Manual*, 2nd. ed., Cold Spring Harbor Press, 1989), followed by Western blot analysis using anti-ricin antibodies (Sigma).
- 20 Cathepsin B may be obtained from Medcor or Calbiochem. Matrix metalloproteinases may be prepared substantially as described by Lark, M.W. et al. (*Proceedings of the 4th International Conference of the Inflammation Research Association* Abstract 145 (1988)) and Welch, A.R. et al. (*Arch. Biochem. Biophys.* 324:59-64 (1995)). Candida acid protease may be
- 25 prepared substantially as described in Remold, H.H. et al. (*Biochim. Biophys. Acta* 167:399-406 (1968)), Ray, T.L. and Payne, C.D. (*Infect. Immunol.* 58:508-514 (1990)) and Fusek, M. et al. (*FEBS Lett.* 327:108-112 (1993)). Hepatitis A protease may be prepared as described in Jewell, D.A. et al. (*Biochemistry* 31:7862-7869 (1992)). Plasmodium proteases may be prepared as described
- 30 in Goldberg, D.E. et al. (*J. Exp. Med.* 173:961-969 (1991)) and Cooper, J.A. and Bujard, H. (*Mol. Biochem. Parasitol.* 56:151-160 (1992)).

#### **In Vitro Cytotoxicity Assay:**

Human ovarian cancer cells (e.g. MA148) are seeded in 96-well flat-bottom plates and are exposed to ricin-like toxin variants or control

medium at 37°C for 16 h. The viability of the cancer cells is determined by measuring [<sup>35</sup>S]methionine incorporation and is significantly lower in wells treated with the toxin variants than those with control medium.

In Vivo Tumour Growth Inhibition Assay:

- 5 Human breast cancer (e.g. MCF-7) cells are maintained in suitable medium containing 10% fetal calf serum. The cells are grown, harvested and subsequently injected subcutaneously into ovariectomized athymic nude mice. Tumour size is determined at intervals by measuring two right-angle measurements using calipers. In animals that received ricin-  
10 like toxin variants containing the matrix metalloproteinase-sensitive linkers, tumour size and the rate of tumour growth are lower than animals in the control group.

In Vivo Tumour Metastasis Assay:

- The metastasis study is performed substantially as described in  
15 Honn, K.V. et al. (*Biochem. Pharmacol.* 34:235-241 (1985)). Viable B16a melanoma tumour cells are prepared and injected subcutaneously into the left axillary region of syngeneic mice. The extent of tumour metastasis is measured after 4 weeks. The lungs are removed from the animals and are fixed in Bouin's solution and macroscopic pulmonary metastases are  
20 counted using a dissecting microscope. In general without therapeutic intervention, injection of 10<sup>5</sup> viable tumour cells forms approximately 40-50 pulmonary metastases. The number of metastases in animal treated with proricin variants containing cathepsin B-sensitive linkers is substantially lower.

25 EXAMPLE 4

In Vitro Protease Digestion of Proricin Variants by Cancer Proteases Cathepsin B or MMP-9

The general protocol for proricin digestion by cancer proteases is described in Examples 2 and 3.

30 In Vitro Protease Digestion of Cathepsin B Proricin Variant

Affinity-purified mutant proricin is treated with individual disease-specific proteases to confirm specific cleavage in the linker region. The proricin substrate is digested in a Cathepsin B protease buffer (50 mM Sodium acetate, 2 mM EDTA, 0.05% Triton) at 40°C. Two hours and

overnight (16 hr) digestion reactions are carried out using 100ng of proricin substrate and 100 and 618 ng of Cathepsin B protease per reaction (CALBIOCHEM, USA). The cleavage products of proricin (ricin A and B chains) are identified using SDS/PAGE (Sambrook et al., Molecular cloning: a laboratory Manual, 2nd. ed., Cold Spring Harbor Press, 1989), followed by Western blot analysis using anti-ricin antibodies (Sigma).

#### In Vitro Protease Digestion of MMP-9 Proricin Variant

Affinity-purified mutant proricin is treated with individual disease-specific proteases to confirm specific cleavage in the linker region. The proricin substrate is digested in 1X column buffer (100 mM NaCl, 50 mM Tris, PH 7.5) at 37°C. Two hours and overnight (16 hr) digestion reactions are set up using 50 ng of MMP-9 proricin substrate and 20 and 200 ng of MMP-9 protease per reaction (CALBIOCHEM, USA). The cleavage products of proricin (ricin A and B chains) are identified using SDS/PAGE (Sambrook et al., Molecular cloning: a laboratory Manual, 2nd. ed., Cold Spring Harbor Press, 1989), followed by Western blot analysis using anti-ricin antibodies (Sigma).

The protocol for Western analysis of ricin chains is described in Example 2.

#### Results

Figures 48 and 49 illustrate Western blots showing the cleavage of the protease-sensitive linkers by cathepsin B (pAP 214) and MMP-9 (pAP 220) respectively. Without protease digestion, the proricin variant appears as a single band at approximately 60 kDa (Lane B of Figure 48 and Lane A of Figure 49). Wild type ricin A chain and B chain appear as two disparate bands at approximately 30 kDa (Lane A of Figure 48 and Lane E of Figure 49). Increasing extent of proricin cleavage can clearly be observed with increasing protease concentration (Lanes C and D of Figure 48 and Lanes B-C of Figure 49).

#### EXAMPLE 5

In vitro protease digestion of various proricin variants by their corresponding proteases.

The general protocol for proricin digestion by corresponding proteases was as described in Examples 2 and 3 and should be considered in connection with the digestions described below.

**Cleavage of pAP-222 protein with the Matrix Metalloproteinase 2**

5 **(MMP-2)**

Affinity-purified mutant proricin is treated with individual disease-specific proteases to confirm specific cleavage in the linker region.

The pAP-222 protein sample (1.0 ug) was digested with the MMP-2 protease (1.0 ug) overnight at 37° C. The total volume of the digestion  
10 reaction was 21.5 ul, and 0.250 ug of the reaction sample was loaded on a protein gel. The MMP-2 protease was purchased from Calbiochem-Novabiochem Corporation, USA.

**Cleavage of pAP-248 protein with the Human Cytomegalovirus (HCMV) protease**

15 Affinity-purified mutant proricin is treated with individual disease-specific proteases to confirm specific cleavage in the linker region.

The pAP-248 protein sample (1.19 ug) was digested with the HCMV protease (1.13 ug) overnight at 37°C. The total volume of the digestion was 10.5 ul, and 0.279 ug of the reaction sample was loaded on a protein gel.  
20 The HCMV was purchased from BACHEM Bioscience Inc., USA.

**Cleavage of pAP-256 protein with the Hepatitis A virus 3C (HAV 3C) protease**

Affinity-purified mutant proricin is treated with individual disease-specific proteases to confirm specific cleavage in the linker region.

25 The pAP-256 protein sample (1.26 ug) was digested with the HAV 3C protease (5 ug) overnight at 37°C. The total volume of the digestion was 12.5 ul, and 0.302 ug of the digestion sample was loaded on a protein gel. The HAV 3C protease was a gift from Dr. G. Lawson from Bates Collage, Main, USA.

30 **Cleavage of pAP-270 protein with the Matrix Metalloproteinase 2 (MMP-2)**

Affinity-purified mutant proricin is treated with individual disease-specific proteases to confirm specific cleavage in the linker region.

The pAP-270 protein sample (0.120 ug) was digested with the MMP-2 protease (0.25 ug) overnight at 37° C. The total volume of the digestion reaction was 22.5 ul, and 0.106 ug of the reaction sample was loaded on a protein gel. The MMP-2 protease was purchased from  
5 Calbiochem-Novabiochem Corporation, USA.

**Cleavage of pAP-288 protein with tPA plasminogen tissue activator**

Affinity-purified mutant proricin is treated with individual disease-specific proteases to confirm specific cleavage in the linker region. The pAP-288 protein sample (1.65 ug) was digested with the t-PA protease (0.5  
10 ug) overnight at 37° C. The total volume of the digestion reaction was 55 ul, and 0.6 ug of the reaction sample was loaded on a protein gel. The t-PA was purchased from Sigma Chemical Co., USA.

**Cleavage of pAP-294 protein with human neutrophil elastase**

Affinity-purified mutant proricin is treated with individual disease-specific proteases to confirm specific cleavage in the linker region.  
15 The pAP-256 protein sample (0.6 ug) was digested with the Elastase protease (5 ug) at 25° C for one hour. The total volume of the digestion reaction was 52.5 ul, and 0.171 ug of the digestion sample was loaded on a protein gel. The Human Neutrophil Elastase protease was purchased from  
20 Cedarlane Laboratories Limited, Canada.

**Cleavage of pAP-296 protein with calpain**

Affinity-purified mutant proricin is treated with individual disease-specific proteases to confirm specific cleavage in the linker region. The pAP-296 protein sample (2.05 ug) was digested with the Calpain protease  
25 (10 ug) overnight at 37° C. The total volume of the digestion reaction was 35 ul and 0.761 ug of the reaction sample was loaded on a protein gel. The Calpain protease was purchased from Sigma Chemical Co., USA

**Results**

Figures 52, 54, 58 & 66(MMP-2), 60, 64 and 62 show the cleavage of  
30 proteases of linkers by HCMV, HAV 3C, MMP-2, t-PA, calpain, and human neutrophil elastase respectively. Without protease digestion, the proricin variants appear as a single band at approximately 60kDA (Lane A in connection with Figure 52; Lane B of Figure 54; Lane A of Figure 58; Lane B of Figure 60; and Lane C of Figure 62; lane B of Figure 64 and lane B of

Figure 66). Wild type ricin chain A and B appear as two bands at approximately 30kDA (see for example Lanes C and D of Figure 52) proricin cleavage can clearly be observed with the appearance of 30kDA bands in connection with the protein which has been digested by the  
5     respective protease (see Lane B of Figure 52; Lane C of Figure 54; or Lane B of Figure 58 for examples).

#### **EXAMPLE 6**

##### **In Vitro Translation Assay (Activation by Cancer Proteases Cathepsin B or MMP-9)**

10         The general protocol for the rabbit reticulocyte lysate reaction to test the cytotoxicity of cancer protease-activated proricin is described briefly in Example 3 and is described in more detail in Example 2.

#### **Results**

Activation of pAP 214 and pAP 220 proricin variants by cathepsin B  
15     and MMP-9, based on the method of May et al. (EMBO J. 8:301-308, 1989), is illustrated in Figures 50 and 51 respectively. The appearance of the 390 base pair product (positive control) is observed in Lane F of Figure 50 and Lane G of Figure 51. This 390 base pair product is absent in the negative control lanes. Without cathepsin or MMP-9 activation, no or minimal N-glycosidase activity in the pAP 214 variant (Lanes H to L, Figure 50) or the  
20     pAP 220 variant (Lanes A to E, Figure 51) was observed. When the pAP 214 variant and the pAP 220 variant were activated by cathepsin or MMP-9 respectively, appearance of the 390 base pair product was observed in a proricin concentration-dependent manner (Lanes A to E of Figure 50 and  
25     Lanes H to L of Figure 51). The present experimental series demonstrated the successful and selective activation of proricin variants by cancer-associated proteases.

#### **EXAMPLE 7**

30         The general protocol for the rabbit reticulocyte lysate reaction is described briefly in Example 3 and is described in more detail in Example 2, all of which compliments the description below.

##### **Depurination of Rabbit Reticulocyte 28S Ribosomal RNA by Digested and Undigested Ricin Variants**



Affinity-purified mutant proricin mutants which were previously digested with the disease-specific protease, were reduced with 5% 2-mercaptoethanol then diluted to 100ng, 14.2ng, 2.0ng, 291pg, and 41.7pg with 1 X ENDO buffer (25mM Tris pH 7.6, 25mM KCl, 5mM MgCl<sub>2</sub>) and  
5 incubated with rabbit reticulocyte lysate, untreated (Promega) for 30 minutes at 30°C. To compare the digested with the undigested proricin variant, the proricin in digestion buffer (according to the specific digestion protocol) was treated in the same manner as the digested sample. As a positive and negative control, 10ng of ricin A chain and 1 X ENDO buffer  
10 consecutively, was incubated with rabbit reticulocyte lysate, untreated, for 30 min at 30°C.

#### **Aniline Cleavage of rRNA and Gel Fractionation**

Total RNA was then extracted from reticulocyte lysate translation mixtures with Trizol reagent (Gibco-BRL) as per manufacturer's  
15 instructions. The RNA was incubated with 80ul of 1M aniline (distilled) with 2.8M acetic acid for 3 min at 60°C in the dark. Ethanol-precipitated RNA samples were dissolved in 20ul of 50% formamide, 0.1X E buffer (3.6mM Tris, 3mM NaH<sub>2</sub>PO<sub>4</sub>, 0.2mM EDTA), and 0.05% xylene cyanol. 10ul of this was heated to 70°C for 2 minutes, loaded and electrophoresed  
20 in 1.2% agarose, 0.1X E buffer, and 50% formamide gel with RNA running buffer (0.1 X E buffer, 0.2% SDS).

#### **Results**

Activation of pAP-248 proricin variant by HCMV; pAP-256 by HAV3C protease; pAP-270 by MMP-2 protease; pAP-288 by t-PA protease;  
25 pAP-294 by human neutrophil elastase; pAP-296 by calpain; and pAP-222 by MMP-2 is illustrated in Figures 52, 55, 59, 61, 63, 65, and 67 respectively. The appearance of the 390 base pair product (deposit of control) is observed in lane L of Figures 53, 55, 61, 63, 65 and 67. The 390 base pair product is observed in lane A of Figures 59 (activation of pAP-270 by  
30 MMP-2). This 390 base pair product is absent in the negative control lanes. Without the specific protease activation, no or minimal activity is seen in the lanes which contained only the proricin variant without digestion (see lane A, B, C, D, and E of Figures 53, 55, 61, 63, 65, and 67). The same observation is made in connection with pAP-270 in Figure 59, however, the

undigested lanes appear as H, I, J, K and L. When the variant was activated by its respective protease, there is an appearance of the 390 base pair product in a proricin concentration-dependent manner (see Lanes H, I, J, K and L of Figure 53, 55, 61, 63, 65, and 67 and Lanes A, B, C, D, and E of Figure 59). The present experimental series demonstrate the successful and selective activation of the identified proricin variants by selective corresponding proteases.

#### **EXAMPLE 8**

#### **Procedure for Examining the Cytotoxicity of Ricin and Ricin Variants on the COS-1 Cell Line**

##### **Cell Preparation**

After washing with 1XPBS (0.137 M NaCl, 2.68 mM KCl, 8.10 mM  $\text{Na}_2\text{HPO}_4$ , 1.47 mM  $\text{KH}_2\text{PO}_4$ ), cells in log phase growth were removed from plates with 1X trypsin/EDTA (Gibco/BRL). The cells were centrifuged at 1100 rpm for 3 min, resuspended in Dulbecco's Modified Eagle Medium containing 10%FBS and 1X pen/strep, and then counted using a haemocytometer. They were adjusted to a concentration of  $5 \times 10^4$  cells $\cdot\text{ml}^{-1}$ . One hundred microliters per well of cells was added to wells 2B - 2G through to wells 9B - 9G of a Falcon 96 well tissue culture plate. A separate 96 well tissue culture plate was used for each sample of Ricin or Ricin variant. The plates were incubated at 37°C with 5%  $\text{CO}_2$  for 24 hours.

##### **Toxin Preparation**

The Ricin and Ricin variants were sterile filtered using a 0.22 $\mu\text{m}$  filter (Millipore). The concentration of the sterile samples were then quantified by  $A_{280}$  and confirmed by BCA measurements (Pierce). For the variants digested with the protease in vitro, the digests were carried out as described in the digestion procedure for each protease. The digests were then diluted in the 1000 ng $\cdot\text{ml}^{-1}$  dilution and sterile filtered. The Ricin and the undigested pAP214 in the pAP 214 cytotoxicity data were treated in the same manner but without the Cathepsin B treatment. Ricin and Ricin variants were serially diluted to the following concentrations: 1000 ng $\cdot\text{ml}^{-1}$ , 100 ng $\cdot\text{ml}^{-1}$ , 10 ng $\cdot\text{ml}^{-1}$ , 1 ng $\cdot\text{ml}^{-1}$ , 0.1 ng $\cdot\text{ml}^{-1}$ , 0.01 ng $\cdot\text{ml}^{-1}$ , 0.001 ng $\cdot\text{ml}^{-1}$  with media containing 10%FBS and 1X pen/strep.

##### **Application of Toxin or Variants to Plates**

Columns 2 to 9 were labeled: control, 1000 ng•ml<sup>-1</sup>, 100 ng•ml<sup>-1</sup>, 10 ng•ml<sup>-1</sup>, 1 ng•ml<sup>-1</sup>, 0.1 ng•ml<sup>-1</sup>, 0.01 ng•ml<sup>-1</sup>, 0.001 ng•ml<sup>-1</sup> consecutively. The media was removed from all the sample wells with a multichannel pipettor. For each plate of variant and toxin, 50µl of media was added to wells 2B to 2G as the control, and 50µl of each sample dilution was added to the corresponding columns. For the pAP220 + MMP-9 data, the plates were incubated for one hour at 37(C with 5% CO<sub>2</sub>, then washed once and replaced with media, then incubated for 48 hours at 37(C with 5% CO<sub>2</sub>. For the pAP 214 + Cathepsin B data, the toxin was left on the plates and incubated for 24 hours at 37(C with 5% CO<sub>2</sub>, then 50 µl of media was added to the wells with the toxin and incubated for another 24 hours at 37(C with 5% CO<sub>2</sub>.

#### **Sample Application**

The whole amount of media (and/or toxin) was removed from each well with a multichannel pipettor, and replaced with 100 µl of the substrate mixture (Promega Cell Titer 96 Aqueous Non-Radioactive Cell Proliferation Assay Kit). The plates were incubated at 37(C with 5% CO<sub>2</sub> for 2 to 4 hours, and subsequently read with a Spectramax 340 96 well plate reader at 490nm. The IC<sub>50</sub> values were calculated using the GRAFIT software program.

#### **Results**

In experiments with pAP-214 and Cathepsin B incubated with COS-1 cells, it may be seen that cells incubated with pAP-214 alone, pAP-214 was ineffective at causing cell death (see Figure 56). However, the cytotoxicity of pAP-214 digested with Cathepsin B behaves similarly to the ricin control in COS-1 cells. This is also illustrated in Figure 56. Similarly, the cytotoxicity of undigested pAP-220 when incubated with COS-1 cells is lower than the cytotoxicity observed with COS-1 cells incubated with pAP-220 digested with MMP-9. Indeed the results suggest that the toxicity of digested pAP-220 is greater than that of ricin. (See Figure 57).

#### **EXAMPLE 9**

#### **Procedure for Examining the Cytotoxicity of Ricin and Ricin Variants on Various Tissue Culture Cell Lines**

### **Cell Preparation**

After washing with 1XPBS (1.37M NaCl, 26.8mM KCl, 81mM Na<sub>2</sub>HPO<sub>4</sub>, 14.7mM KH<sub>2</sub>PO<sub>4</sub>), cells in log phase growth were removed from plates with 1X trypsin/EDTA (Gibco/BRL). The cells were  
5 centrifuged at 1100 rpm for 3 min, resuspended in media containing 10%FBS and 1X pen/strep (media used depended on the cell line being tested), and then counted using a haemocytometer. They were adjusted to a concentration of  $5 \times 10^4$  cells•ml<sup>-1</sup> (faster growing cell lines were adjusted to  $2 \times 10^4$  cells•ml<sup>-1</sup>). One hundred microliters per well of cells  
10 was added to wells 2B - 2G through to wells 9B - 9G of a Falcon 96 well tissue culture plate. A separate 96 well tissue culture plate was used for each sample of Ricin or Ricin variant. The plates were incubated at 37(C with 5% CO<sub>2</sub> for 24 hours.

### **Toxin Preparation**

15 The Ricin and Ricin variants were sterile filtered using a 0.22µm filter (Millipore). The concentration of the sterile samples were then quantified by A<sub>280</sub> and confirmed by a BCA measurement (Pierce). Ricin and Ricin variants were serially diluted to the following concentrations: 3000 ng•ml<sup>-1</sup>, 300 ng•ml<sup>-1</sup>, 30 ng•ml<sup>-1</sup>, 3 ng•ml<sup>-1</sup>, 0.3 ng•ml<sup>-1</sup>,  
20 0.03ng•ml<sup>-1</sup>, 0.003 ng•ml<sup>-1</sup> with media containing 10%FBS and 1X pen/strep.

### **Application of Toxin or Variants to Plates**

Columns 2 to 9 were labeled: control, 0.001 ng•ml<sup>-1</sup>, 0.01 ng•ml<sup>-1</sup>, 0.1 ng•ml<sup>-1</sup>, 1ng•ml<sup>-1</sup>, 10 ng•ml<sup>-1</sup>, 100 ng•ml<sup>-1</sup>, 1000 ng•ml<sup>-1</sup>  
25 consecutively. For each plate of variant and toxin, 50µl of media was added to wells 2B to 2G as the control, and 50µl of each sample dilution was added to the corresponding columns containing 100µl per well of cells (i.e. 50 µl of the 3000 ng•ml<sup>-1</sup> dilution added to the wells B-G in column 9, labeled 1000 ng•ml<sup>-1</sup>). The plates were incubated for 48 hours at 37(C  
30 with 5% CO<sub>2</sub>.

### **Sample Application**

An amount of 140µl was removed from each well with a multichannel pipettor, and replaced with 100 µl of the substrate mixture

(Promega Cell Titer 96 Aqueous Non-Radioactive Cell Proliferation Assay Kit). The plates were incubated at 37°C with 5% CO<sub>2</sub> for 2 to 4 hours, and subsequently read with a Spectramax 340 96 well plate reader at 490nm. The IC<sub>50</sub> values were calculated using the GRAFIT software program.

## 5 **Results**

Referring to Table 2, it may be seen that the survival of cells is correlated with the proricin variant and the cell specific protease produced by the cell type. For example, in the HT1080 cell line, both pAP-214 and pAP-220 required only 2-1/2 times the amount of ricin to achieve the same level of cytotoxicity. On the other hand, pAP-224 required 193 times the amount of ricin to achieve the same level of cell death. As well, it may be seen that in the cells where expression of Cathepsin D is found, pAP-214 and 220 were more effective at causing cell death than ricin and more effective than pAP-224. Details concerning the various cells types used in these experiments are outlined below.

### **COS-1 (African Green Monkey Kidney Cells)**

This is an SV40 transformed cell line which was prepared from established simian cells CV-1. (Reference: Gluzman, Y. (1975) Cell, 23, 175 - 182)(ATCC CRL 1650)

### 20 **HT-1080 Human Fibrosarcoma**

(ATCC CCL 121) This cell line was shown to produce active MMP-9 in tissue culture. References: Moore et al. (1997) Gynecologic Oncology 65, 83-88.

### **9L Rat Glioblastoma**

25 Glioblastomas are generally associated with cathepsin B expression. Levels of cathepsin B expression correspond to the extent of progression of malignancy i.e. highest levels for glioblastomas over anaplastic astrocytomas over low-grade gliomas and normal brain tissue. The 9L cell line was provided by Dr. William Jia of the B.C. Cancer Agency.

30 References: Mikkelsen et al. (Aug. 1995) Journal of Neurosurgery 83(2), 285-290. Nakano et al. (1995) J. of Neurosurgery 83(2), 298-307.

### **MCF-7 Human Breast Cancer Cell Line (Epithelial)**

(ATCC CRL 1555) In the absence of estrogen cathepsin B has not been shown to be elevated relative to normal cells. It can be induced with estrogen to produce Cathepsin D. Production of MMP-9 is unknown.

5 Having illustrated and described the principles of the invention in a preferred embodiment, it should be appreciated to those skilled in the art that the invention can be modified in arrangement and detail without departure from such principles. We claim all modifications coming within the scope of the following claims.

10 All publications, patents and patent applications referred to herein are incorporated by reference in their entirety to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated by reference in its entirety.

Bever Jr., C.T., Panitch, H.S., and Johnson, K.P. (1994) *Neurology* 44(4), 745-8. Increased cathepsin B activity in peripheral blood mononuclear cells of multiple sclerosis patients.

Cohen, P., Graves, H.C., Peehl, D.M., Kamarei, M., Giudice, L.C., and Rosenfeld, R.G. (1992) Journal of Clinical Endocrinology and Metabolism 75(4), 1046-53. Prostate-specific antigen (PSA) is an insulin-like growth factor binding protein-3 protease found in seminal plasma.

- 10 Conover, C.A. and De Leon, D.D. (1994) J. Biol. Chem. 269(10), 7076-80.  
Acid activated insulin-like growth factor-binding protein-3 proteolysis in  
normal and transformed cells. Role of cathepsin D.

Hansen, G., Schuster, A., Zubrod, C., and Wahn, V. (1995) Respiration  
62(3), 117-24. Alpha 1-proteinase inhibitor abrogates proteolytic and  
15 secretagogue activity of cystic fibrosis sputum.

Muller, H.L., Oh, Y., Gargosky, S.E., Lehrnbecher, T., Hintz, R.L., and Rosenfeld, R.G. (1993) Journal of Clinical Endocrinology and Metabolism 77(5), 1113-9. Concentrations of insulin-like growth factor (IGF)-binding protein-3 (IGFBP-3), IGF, and IGFBP-3 protease activity in cerebrospinal fluid of children with leukemia, central nervous system tumor, or meningitis.

TABLE 1

Table 1 - Sequence and Location of Oligonucleotide Primers

Name of Primer	Primer Sequence †	Corresponds to preproricin nucleotide numbers: (see Figures 8-10)
Ricin-109	5' - GGAGATGAAACCGGGAGGAAATACTATTGTAAT-3'	27 to 59
Ricin-99Eco	5' - <u>GCGGAATT</u> CCGGGAGGAAATACTATTGTAAT -3'	37 to 59
Ricin267	5' - ACGGTTTATTTTAGTTGA-3'	300 to 317
Ricin486	5' - ACTTGCTGGTAATCTGAG -3'	519 to 536
Ricin725	5' - AGAATAGTTGGGGGAGAC -3'	758 to 775
Ricin937	5' - AATGCTGATGTTTGTATG -3'	970 to 987
Ricin1151	5' - CGGGAGTCTATGTGATGA -3'	1184 to 1201
Ricin1399	5' -GCAAATAGTGGACAAGTA -3'	1432 to 1449
Ricin 1627	5' - GGATTGGTGTTAGATGTG -3'	1660 to 1677
Ricin1729C	5' - ATAACCTGCTGTCCTTTCA -3'	1864 to 1846
Ricin1729C Xba	5' - <u>CGCTCTAG</u> ATAACTTGCTGTCCTTTCA	1864 to 1846

† Underlined sequences inserted for subcloning purposes and not included in final preproricin sequences

00440-04400



<b><u>Cell Line</u></b>	<b><u>IC50<sub>Ricin</sub></u> <b>(ng/ml)</b></b>	<b><u>IC50<sub>pAP214</sub></u> <b>IC50<sub>Ricin</sub></b></b>	<b><u>IC50<sub>pAP220</sub></u> <b>IC50<sub>Ricin</sub></b></b>	<b><u>IC50<sub>pAP224</sub></u> <b>IC50<sub>Ricin</sub></b></b>
COS-1	0.1	17	22	150
HT1080	0.5	2.46	2.14	193
9L	10.8	1.3	1.7	32.3
MCF-7 (without estrogen)	0.09	27.8	40	742

**I CLAIM:**

1. A purified and isolated nucleic acid having a nucleotide sequence encoding an A chain of a ricin-like toxin, a B chain of a ricin-like toxin and a heterologous linker amino acid sequence linking the A and B chains, the  
5 heterologous linker sequence containing a cleavage recognition site for a protease localized in cells or tissues affected by a specific disease.
2. The nucleic acid sequence of claim 1 wherein the linker sequence encodes a peptide wherein at least 5 amino acids differ from the wild-type.
3. The nucleic acid sequence of claim 2 wherein the linker sequence  
10 contains a cleavage recognition site recognized by a protease selected from the group consisting of: a cancer associated protease, a viral protease, a fungal protease, and a parasite protease.
4. A nucleic acid sequence of claim 3 wherein the A chain is ricin A chain, abrin toxin A chain, diphtheria toxin A chain, or Domain I of  
15 Pseudomonas exotoxin.
5. A nucleic acid molecule of claim 3 wherein the A chain is volkensin toxin A chain, cholera toxin A chain, modeccin toxin A chain, viscumin toxin A chain or shiga toxin A chain.
6. A nucleic acid sequence of claim 3 wherein the B chain is ricin B  
20 chain, abrin toxin A chain, diphtheria toxin B chain, or Domain II/III of Pseudomonas exotoxin.
7. A nucleic acid molecule of claim 3 wherein the B chain is volkensin toxin B chain, cholera toxin B chain, modeccin toxin B chain, viscumin toxin B chain or shiga toxin B chain.
- 25 8. A nucleic acid sequence of claim 3 wherein the cleavage recognition site is recognized by a cancer-associated protease which is selected from

the group consisting of: cathepsin B, a matrix metalloproteinase, cathepsin L, cathepsin D, urokinase-type plasminogen activator, tissue-type plasminogen activator, human prostate-specific antigen, kallikrein, neutrophil elastase, and calpain.

- 5    9.        A nucleic acid sequence of claim 3 wherein the cleavage recognition site is recognized by a parasitic protease which is a *Plasmodium falciparum* protease.

- 10    10.       A nucleic acid sequence of claim 3 wherein the cleavage recognition site is recognized by viral protease which is selected from the group consisting of: human cytomegalovirus, human herpes virus, varicella zoster virus, hepatitis A virus, hepatitis C virus, Epstein-Barr virus-specific protease, and infectious laryngotracheitis virus.

11.       A nucleic acid sequence of claim 3 wherein the cleavage recognition site is recognized by fungal protease which is a *Candida* acid protease.

- 15    12.       A nucleic acid sequence of claim 3 having the nucleotide sequence according to SEQ ID No. 3; SEQ ID No 5; SEQ ID No 7; SEQ ID No 9; SEQ ID No 11; SEQ ID No 13; SEQ ID No 15; SEQ ID No 17; SEQ ID No 19; SEQ ID No 21; SEQ ID No 23; SEQ ID No 25; SEQ ID No 27; SEQ ID No 29; SEQ ID No 31; SEQ ID No 33; SEQ ID No 35; SEQ ID No 37; SEQ ID No 39; 20    SEQ ID No 48; SEQ ID No 50; SEQ ID No 52; SEQ ID No 54; SEQ ID No 74; SEQ ID No 77; SEQ ID No 80; SEQ ID No 83; SEQ ID No 86; SEQ ID No 89; SEQ ID No 92; SEQ ID No 95; SEQ ID No 98; SEQ ID No 101; SEQ ID No 104; SEQ ID No 107; SEQ ID No 110; SEQ ID No 113; SEQ ID No 116; SEQ ID No 119; SEQ ID No 122; or SEQ ID No 125.

- 25    13.       A plasmid incorporating the nucleic acid of claim 12.

14.       A baculovirus transfer vector incorporating the nucleic acid of claim 12.

15. A recombinant protein comprising an A chain of a ricin-like toxin, a B chain of a ricin-like toxin and a heterologous linker amino acid sequence, linking the A and B chains, wherein the linker sequence contains a cleavage recognition site for a protease localized in cells or tissues affected by a specific disease.
16. The recombinant protein of claim 15 wherein at least 5 amino acids of the linker amino acid sequence differ from the wild-type.
17. The recombinant protein of claim 16 wherein the linker sequence contains a cleavage recognition site which is recognized by a protease selected from the group consisting of: a cancer, viral, fungal, and a parasitic protease.
18. A recombinant protein of claim 17 wherein the A chain is ricin A chain, abrin toxin B chain, diphtheria toxin A chain, or Domain I of Pseudomonas exotoxin.
19. A recombinant protein of claim 17 wherein the A chain is volkensin toxin A chain, cholera toxin A chain, modeccin toxin A chain, viscumin toxin A chain, or shiga toxin A chain.
20. A recombinant protein of claim 17 wherein the B chain is ricin B chain, abrin toxin B chain, diphtheria toxin B chain, or Domain II/III of Pseudomonas exotoxin.
21. A recombinant protein of claim 17 wherein the B chain is volkensin toxin B chain, cholera toxin B chain, modeccin toxin B chain, viscumin toxin B chain, or shiga toxin B chain.
22. A recombinant protein of claim 17 wherein the cleavage recognition site is recognized by a cancer-associated protease selected from the group consisting of: cathepsin B, a matrix metalloproteinase, cathepsin L, cathepsin D, urokinase-type plasminogen activator, tissue-type

plasminogen activator, human prostate-specific antigen, kallikrein, neutrophil elastase, and calpain.

23. A recombinant protein of claim 17 wherein the cleavage recognition site is recognized by a parasitic protease which is a  
5 Plasmodium falciparum protease.

24. A recombinant protein of claim 17 wherein the cleavage recognition site is recognized by a viral protease which is selected from the group consisting of: human cytomegalovirus, human herpes virus, varicella zoster virus, hepatitis A virus, hepatitis C virus, Epstein-Barr  
10 virus-specific protease, and infectious laryngotracheitis virus.

25. A recombinant protein of claim 17 wherein the cleavage recognition site is recognized by a fungal protease which is a Candida acid protease.

26. A recombinant protein of claim 17 having the linker amino acid  
15 sequence according to SEQ ID No. 40; SEQ ID No. 41; SEQ ID No. 42; SEQ ID No. 43; SEQ ID No. 44; SEQ ID No. 45; SEQ ID No. 46; SEQ ID No. 55; SEQ ID No. 56; SEQ ID No. 57; SEQ ID No. 58; SEQ ID No. 59; SEQ ID No. 60; SEQ ID No. 61; SEQ ID No. 62; SEQ ID No. 63; SEQ ID No. 64; SEQ ID No. 65; SEQ ID No. 66; SEQ ID No. 67; SEQ ID No. 68; SEQ ID No. 69; SEQ  
20 ID No. 70; SEQ ID No. 71; SEQ ID No. 72; SEQ ID No. 75; SEQ ID No. 78; SEQ ID No. 81; SEQ ID No. 84; SEQ ID No. 87; SEQ ID No. 90; SEQ ID No. 93; SEQ ID No. 96; SEQ ID No. 99; SEQ ID No. 102; SEQ ID No. 105; SEQ ID No. 108; SEQ ID No. 111; SEQ ID No. 114; SEQ ID No. 117; SEQ ID No. 120; SEQ ID No. 123; or SEQ ID No. 126.

25 27. A method of inhibiting or destroying cells of a tissue affected by a disease, wherein a protease specific to the disease is localized in the cells or tissue comprising the steps of:

(a) preparing a purified and isolated nucleic acid having a nucleotide sequence encoding an A chain of a ricin-like toxin, a B chain of a

ricin-like toxin, and a heterologous linker amino acid sequence, linking the A and B chains, wherein the linker sequence contains a cleavage recognition site for the protease;

(b) introducing the nucleic acid into a host cell and expressing the  
5 nucleic acid in the host cell to obtain a recombinant protein comprising an  
A chain of a ricin-like toxin, a B chain of a ricin-like toxin and a linker amino  
acid sequence;

(c) suspending the protein in a pharmaceutically acceptable carrier, diluent or excipient, and

10 (d) contacting the cells with the recombinant protein.

28. A method according to claim 27 wherein the linker sequence encodes a peptide wherein at least 5 amino acids differ from the wild-type.

29. The method of claim 28 where the disease is one of cancer, a fungal infection, or cells infected with a virus or parasite.

15 30. A method of inhibiting or destroying cells of a tissue affected by a disease, wherein a protease specific to the disease is localized in the cells or tissue comprising the step of contacting the cells with a recombinant protein according to claim 26.

31. A method of treating a disease comprising administering a  
20 recombinant protein according to claim 26 to an animal in need thereof.

32. A method of treating a disease comprising administering a nucleic acid molecule according to claim 12 to an animal in need thereof.

33. A method of treating a mammal with cancer or infected with a fungus, virus or parasite, comprising the steps of preparing a recombinant protein of claim 15 wherein the linker sequence contains a cleavage recognition site for a cancer, fungal, viral or parasitic protease and administering the protein to the mammal.

34. A method of inhibiting or destroying cells affected by a disease associated with a protease, comprising contacting cells affected by a disease associated with the protease with a recombinant ricin protein such that cells affected by a disease associated with a protease are inhibited or  
5 destroyed, wherein said recombinant ricin protein has an A chain of a ricin-like toxin, B-chain of a ricin-like toxin, and a heterologous linker amino acid sequence containing a cleavage recognition site for the protease associated with the disease.

35. A method according to claim 33, wherein said heterologous linker  
10 amino acid sequence contains at least five amino acids which differ from the wild-type.

36. The method of claim 34 where the disease is one of cancer, a fungal infection, or cells infected with a virus or parasite.

37. A process for preparing a pharmaceutical for treating a mammal  
15 with cancer, fungal infection, viral infection or parasitic infection, comprising the steps of :

(a) preparing a purified and isolated nucleic acid having a nucleotide sequence encoding an A chain of a ricin-like toxin, a B chain of a ricin-like toxin, and a heterologous linker amino acid sequence, linking the  
20 A and B chains, wherein the linker sequence contains a cleavage recognition site for a cancer, viral or parasitic protease;

(b) introducing the nucleic acid into a host cell and expressing the nucleic acid in the host cell to obtain a recombinant protein comprising an A chain of a ricin-like toxin, a B chain of a ricin-like toxin and a linker amino  
25 acid sequence;

(c) suspending the protein in a pharmaceutically acceptable carrier, diluent or excipient.

38. A pharmaceutical composition for treating cancer or a fungal, or viral, or parasitic infection in an animal comprising the recombinant

protein of claim 16 and a pharmaceutically acceptable carrier, diluent or excipient.

39. A pharmaceutical composition for treating cancer or a fungal, or viral, or parasitic infection in an animal comprising the nucleic acid molecule of claim 3 and a pharmaceutically acceptable carrier, diluent or excipient.
- 5

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**ABSTRACT OF THE DISCLOSURE**

The present invention provides a protein having an A chain of a ricin-like toxin, a B chain of a ricin-like toxin and a heterologous linker amino acid sequence, linking the A and B chains. The linker sequence  
5 contains a cleavage recognition site for a disease specific protease such as a cancer, fungal, viral or parasitic protease. The invention also relates to a nucleic acid molecule encoding the protein and to expression vectors incorporating the nucleic acid molecule. Also provided is a method of inhibiting or destroying mammalian cancer cells, cells infected with a virus,  
10 a fungus, or parasite, or parasites utilizing the nucleic acid molecules and proteins of the invention and pharmaceutical compositions for treating human cancer, viral infection, fungal infection, or parasitic infection.

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## FIGURE 1

### **Complete Sequence of Baculovirus Transfer Vector, pVL1393**

ID PVL1393 preliminary; circular DNA; SYN;  
9632 BP.  
XX  
AC IG1137;  
XX  
DT 01-FEB-1993 (Rel. 7, Created)  
DT 01-JUL-1995 (Rel. 12, Last updated, Version  
1)  
XX  
DE E. coli plasmid vector pVL1393 - complete.  
XX  
KW cloning vector.  
XX  
OS Cloning vector  
OC Artificial sequences; Cloning vehicles.  
XX  
RN [1]  
RC p2Bac from baculovirus  
RC p2Blue from p2Bac  
RC pBlueBac from AcNPV  
RC pBlueBac2 from AcNPV  
RC pBlueBacIII from AcNPV  
RC pBlueBacHisA from AcNPV  
RC pBlueBacHisB from AcNPV  
RC pBlueBacHisC from AcNPV  
RC pVL1392, pVL1393 from pAc360  
RA ;  
RT ;  
RL The Digest 5:2-2(1992).  
XX  
CC NM (pVL1393)  
CC CM (yes)  
CC NA (ds-DNA)  
CC TP (circular)  
CC ST ()  
CC TY (plasmid)  
CC SP (British  
Biotechnology) (Invitrogen)  
CC HO (E.coli NM522) (E.coli  
INValphaF') (insect)  
CC CP ()  
CC FN (expression) (transfer)  
CC SE ()  
CC PA (pAC360)  
CC BR (pVL1392)  
CC OF ()  
CC OR ()  
XX  
FH Key Location/Qualifiers  
FH

00440" ESTS60

**FIGURE 1 (Cont'd)**

```

FT    misc_feature      0..0
FT                                     /note="1. pAc360, ori/amp/AcMNPV
polyhedrin gene
FT                                     -> pVL1393 9632bp"
FT    transposon        0..0
FT                                     /note="TRN AcMNPV"
FT    misc_binding      868..868
FT                                     /note="SIT SacII"
FT    misc_binding      1395..1395
FT                                     /note="SIT ApaI"
FT    misc_binding      1901..1901
FT                                     /note="SIT XhoI"
FT    promoter          0..0
FT                                     /note="PRO AcMNPV polyhedrin gene"
FT    misc_binding      0..0
FT                                     /note="MCS
FT                                     BamHI-SmaI-XbaI-EcoRI-NotI-XmaIII-PstI-
BglIII"
FT    rep_origin        0..0
FT                                     /note="ORI E. coli pMB1 (ColE1 and
pBR322)"
FT    CDS                complement(0..0)
FT                                     /note="ANT E. coli beta-lactamase gene
(bla)
FT                                     ampicillin resistance gene (apr/amp)"
XX
SQ    Sequence 9632 BP; 2602 A; 2122 C; 2176 G; 2732 T; 0
other;

```

```

aagcttttact cgtaaagcga gttgaaggat catatttagt tgcgtttatg
agataagatt gaaagcacgt gtaaaatggt tcccgcgcggt tggcacaact
atttacaatg cggccaagtt ataaaagatt ctaatctgat atgttttaaa
acacctttgc ggcccgagtt gtttgcgtag gtgactagcg aagaagatgt
gtggaccgca gaacagatag taaaacaaaa ccctagtatt ggagcaataa
tcgattttaac caacacgtct aaatattatg atgggtgtgca ttttttgcggt
gcggggcctgt tatacaaaaa aattcaagta cctggccaga ctttgccgcc
tgaaagcata gttcaagaat ttattgacac ggtaaaagaa tttacagaaa
agtgtcccggt catgttggtg ggcgtgcact gcacacacgg tattaatcgc
accggttaca tgggtgtgcag atattttaatg cacaccctgg gtattgcgcc
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gacaaaatta cgttcaagat ttattaattt aattaatatt atttgcattc
tttaacaaat actttatcct attttcaaat tgttgcgctt cttccagcga
acccaaaacta tgcttcgctt gctccggtta gcttgtagcc gatcagtggc
gttggttccaa tcgacggtag gattaggcgg gatattctcc accacaatgt
tggaacacgtt gatgttacgt ttatgctttt gggtttccac gtacgtcttt
tgcccggtaa tagccgtaaa cgtagtgccg tcgcgcggtca cgcacaacac
cggatgtttg cgcttggtccg cggggtattg aaccgcgcgga tccgacaaat
ccaccacttt ggcaactaaa tcggtgacct gcgcggtcttt tttctgcatt
atttcgtctt tcttttgcac ggtttcctgg aagccggtgt acatgcggtt
tagatcagtc atgacgcgcg tgacctgcaa atctttggcc tcgatctgct
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tcctcggttt tttgcccac caccgcttgc agcgcggttg tgtgctcggt
gaatgtcgca atcagcttag tcaccaactg tttgctctcc tctcccgtt
gtttgatcgc gggatcgtag ttgccggtgc agagcacttg aggaattact
tcttctaaaa gccattcttg taattctatg gcgtaaggca atttggactt

```

**FIGURE 1 (Cont'd)**

cataatcagc	tgaatcacgc	cggatttagt	aatgagcact	gtatgcggct
gcaaatacag	cgggtcgccc	cttttcacga	cgctggtaga	gtaggggcc
ccattttgga	tgggtctgctc	aaataacgat	ttgtatttat	tgtctacatg
aacacgtata	gctttatcac	aaactgtata	ttttaaactg	ttagcgacgt
ccttgggcac	gaaccggacc	tgttggtcgc	gctctagcac	gtaccgcagg
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tttgatacac	gtgtgtcgat	tttgcaacaa	ctattgtttt	ttaacgcaaa
ctaaacttat	tgtggttaagc	aataattaaa	tatgggggaa	catgcgcgcg
tacaacactc	gtcgttatga	acgcagacgg	cgccggtctc	ggcgcaagcg
gctaaaacgt	gttgcgcggt	caacgcggca	aacatcgcaa	aagccaatag
tacagttttg	atgtgcatat	taacgcgcat	tttttaaaatt	atcttattta
ataaatagtt	atgacgccta	caactccccg	cccgcgttga	ctcgtgcac
ctcgagcagt	tcgttgacgc	cttcctccgt	gtggccgaac	acgtcgagcg
ggtggtcgat	gaccagcggc	gtgccgcacg	cgacgcacaa	gtatctgtac
accgaatgat	cgtcgggcga	aggcacgtcg	gcctccaagt	ggcaatattg
gcaaattcga	aaatatatac	agttgggttg	tttgccgata	tctatcgtgg
cgttggggcat	gtacgtccga	acgttgattt	gcatgcaagc	cgaaattaaa
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gccgtcgatt	aaatcgcgca	atcgagtcaa	gtgatcaaag	tgtggaataa
tgttttcttt	gtattcccga	gtcaagcgca	gcgcgtattt	taacaaacta
gccatcttgt	aagttagttt	cattttaatgc	aactttatcc	aataatatat
tatgtatcgc	acgtcaagaa	ttaacaatgc	gcccgttgtc	gcattctaac
acgactatga	tagagatcaa	ataaagcgcg	aattaaatag	cttgcgacgc
aacgtgcacg	atctgtgcac	gcgttcgggc	acgagctttg	attgtaataa
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aaagaactgc	cgactacaaa	attaccgagt	atgtcggtga	cgttaaaact
attaagccat	ccaatcgacc	gttagtcgaa	tcaggaccgc	tgggtcgaga
agccgcgaag	tatggcgaa	gcacgtgata	acgtgtggag	tccgctcatt
agagcgtcat	gtttagacaa	gaaagctaca	tattttaattg	atccccgatga
ttttattgat	aaattgaccc	taactccata	cacggtattc	tacaatggcg
gggttttggt	caaaatttcc	ggactgcat	tgtacatgct	gttaacggct
ccgccacta	ttaatgaaat	taaaaattcc	aatttttaaaa	aacgcagcaa
gagaaacatt	tgtatgaaag	aatgcgtaga	aggaaagaaa	aatgtcgtcg
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aacgatttga	aagaaaacaa	tgtaccgcgc	ggcggtatgt	acaggaagag
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ttgctggcaa	ctgcaagggt	ctcaatccta	tttgtaatta	ttgaataata
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gcaacaagaa	catttgtagt	attatctata	attgaaaacg	cgtagttata
atcgctgagg	taatatttaa	aatcattttc	aaatgattca	cagtttaatt
gcgacaatat	aattttattt	tcacataaac	tagacgcctt	gtcgtcttct
tcttcgtatt	ccttctcttt	ttcatttttc	tcctcataaa	aattaacata
gttattatcg	tatccatata	tgtatctatc	gtatagagta	aattttttgt
tgtcataaat	atatatgtct	tttttaaatg	ggtgtatagt	accgctgcgc
atagtttttc	tgtaatttac	aacagtgcct	ttttctggta	gttcttcgga
gtgtgttgct	tttaattatta	aatttatata	atcaatgaat	ttgggatcgt
cggttttgta	caatatgttg	ccggcatagt		
acgcagcttc	ttctagtcca	attacaccat	tttttagcag	caccggatta
acataacttt	ccaaaatggt	gtacgaaccg	ttaaacaana	acagttcacc
tcccttttct	atactattgt	ctgcgagcag	ttgtttgttg	ttaaaaaata
cagccattgt	aatgagacgc	acaaactaat	atcacaaact	ggaaatgtct

**FIGURE 1 (Cont'd)**

ctgtcccgat	ttatTTgaaa	cactacaaat	taaaggcgag	ctttcgtagc
aacttgtag	caatattatt	agacagctgt	gtgaagcgct	caacgatttg
cacaagcaca	atttcataca	caacgacata	aaactcgaaa	atgtcttata
tttcgaagca	cttgatcgcg	tgtatgtttg	cgattacgga	ttgtgcaaac
acgaaaactc	acttagcggtg	cacgacggca	cgttggagta	tttagtccg
gaaaaaattc	gacacacaa	tatgcacgtt	tcgtttgact	ggtacgcggc
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gcattaatga	atcggccaa	gcgcggggag	aggcggtttg	cgtattgggc
gctcttccgc	ttcctcgctc	actgactcgc	tgcgctcggt	cgttcggctg
cggcgagcgg	tatcagctca	ctcaaaggcg	gtaatacggg	tatccacaga
atcaggggat	aacgcaggaa	agaacatgtg	agcaaaaagg	cagcaaaaagg
ccaggaaccg	taaaaaggcc	gcgttgctgg	cgtttttcca	taggctccgc
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aacccggtaa				
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gcgaggtatg	taggcgggtgc	tacagagttc	ttgaagtggg	ggcctaacta
cggtacact	agaaggacag	tatttggtat	ctgcgctctg	ctgaagccag
ttaccttcgg	aaaaagagtt	ggtagctctt	gatccggcaa	acaaaccacc
gctggtagcg	gtggtttttt	tgtttgcaag	cagcagatta	cgcgcagaaa
aaaaggatct	caagaagatc	ctttgatctt	ttctacgggg	tctgacgctc
agtggaaacga	aaactcacgt	taagggattt	tggtcatgag	attatcaaaa
aggatcttca	cctagatcct	tttaaattaa	aatgaagtt	ttaaatcaat
ctaaagtata	tatgagtaaa	cttggctctga	cagttaccaa	tgtttaatca
gtgaggcacc	tatctcagcg	atctgtctat	ttcgttcata	catagttgcc
tgactccccg	tcgtgtagat	aactacgata	cgggaggggc	taccatctgg
ccccagtgtc	gcaatgatac	cgcgagaccc	acgctcaccg	gctccagatt
tatcagcaat	aaaccagcca	gccggaaggg	ccgagcgcag	aagtggctct
gcaactttat	ccgcctccat	ccagctctatt	aattggttgcc	gggaagctag
agtaagtagt	tcgccagtta	atagtttgcg	caacgttggt	gccattgcta
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ggttcccaac	gatcaaggcg	agttacatga	tcccccatgt	tgtgcaaaaa
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cagtgttatc	actcatggtt	atggcagcac	tgcataattc	tcttactgtc
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ggaaaacggt	cttcggggcg	aaaactctca	aggatcttac	cgctgttgag
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gcaaaaaaag	gaataagggc	gacacggaaa	tgttgaatac	tcataactct
cctttttcca	tattattgaa	gcatttatca	gggttattgt	ctcatgagcg
gatacatatt	tgaatgtatt	tagaaaaata	aacaaatagg	ggttccgcgc
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gacattaacc	tataaaaata	ggcgtatcac	gaggcccttt	cgtctcgcgc
gtttcgggtga	tgacggtgaa	aacctctgac	acatgcagct	cccgagagacg
gtcacagctt	gtctgtaagc	ggatgccggg	agcagacaag	cccgtcaggg

**FIGURE 1 (Cont'd)**

```

atcaatatat agttgctgat atcatggaga taattaaaat gataaccatc
tcgcaaataa ataagtattt tactgttttc gtaacagttt tgtaataaaa
aaacctataa atattccgga ttattcatat cgtcccacca tcgggcgcgg
atcccgggta ccttctagaa ttccggagcg gccgctgcag atctgatcct
ttcctgggac ccggcaagaa ccaaaaactc actctcttca aggaaatccg
taatgttaaa cccgacacga tgaagcttgt cgttggatgg aaaggaaaag
agttctacag ggaaacttgg acccgcttca tggaagacag ctccccatt
gttaacgacc aagaagtgat ggatgttttc cttgttgtea acatgcgtcc
cactagacc aaccgttgtt acaaattcct ggcccaacac gctctgcgtt
gcgaccccgga ctatgtacct catgacgtga ttaggatcgt cgagccttca
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atcggtagcg actctgctga agaggaggaa attctccttg aagtttccct
ggtgttcaaa gtaaaggagt ttgcaccaga cgcacctctg ttcactggtc
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gattctgtgc gttgttgatt tacagacaat tgttgtacgt attttaataa
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ttgtaaaaata ggtttcgatt agttttcaaac aagggttgtt tttccgaacc
gatggctgga ctatctaata gattttcgct caacgccaca aaacttgcca
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tgtaataaag gttcgacgtc gttcaaaaata ttatgcgctt ttgtatttct
ttcatcactg tcgttagtgt acaattgact cgacgtaaac acgttaaata
aagcttggac atatttaaca tcgggcgtgt tagctttatt aggccgatta
tcgtcgtcgt cccaaccctc gtcgttagaa gttgcttccg aagacgattt
tgccatagcc acacgacgcc tattaaattgt gtcggctaac acgtccgcga
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tgggcggggt tcaatctaac tgtgccgat ttttaattcag acaacacgtt
agaaagcgat ggtgcaggcg gtggtaacat ttcagacggc aaatctacta
atggcggcgg tgggtggagc gatgataaat ctaccatcgg tggaggcgca
ggcggggcgt ggcggcgagg cggaggcgga ggtggtggcg gtgatgcaga
cggcggttta ggctcaaatg tctctttagg caacacagtc ggcacctcaa
ctattgtact ggtttcgggc gccgtttttg gtttgaccgg tctgagacga
gtgcgatttt tttcgtttct aatagcttcc aacaattggt gtctgtcgtc
taaaggtgca gcgggttgag gttccgtcgg cattggtgga gcgggcggca
attcagacat cgatgggtgg ggtggtgggt gaggcgctgg aatggtaggc
acgggagaag gtggtggcgg cggtgccgcc ggtataattt gttctggttt
agtttgttcg cgcacgattg tgggcaccgg cgcaggcgcc gctggctgca
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acgtacatgt atgctttgtt gtcaaaaacg tcgttggcaa gctttaaaat
atttaaaaga acatctctgt tcagcaccac tgtgttgteg taaatgttgt
tttgataat ttgcgcttcc gcagtatcga cacgttcaaa aaattgatgc
gcatcaattt tgttgttcc attattgaat aaataagatt gtacagattc
atatctacga ttcgtcatgg ccaccacaaa tgctacgctg caaacgctgg
tacaatttta cgaaaactgc aaaaacgtca aaactcggt taaaataatc
aacgggcgct ttggcaaaat atctatttta tcgcacaagc ccactagcaa
attgtatttg cagaaaacaa tttcggcgca caattttaac gctgacgaaa
taaaagttca ccagttaatg agcgaccacc caaattttat aaaaatctat
tttaatcacg gttccatcaa caaccaagt atcgtgatgg actacattga

```

**FIGURE 1 (Cont'd)**

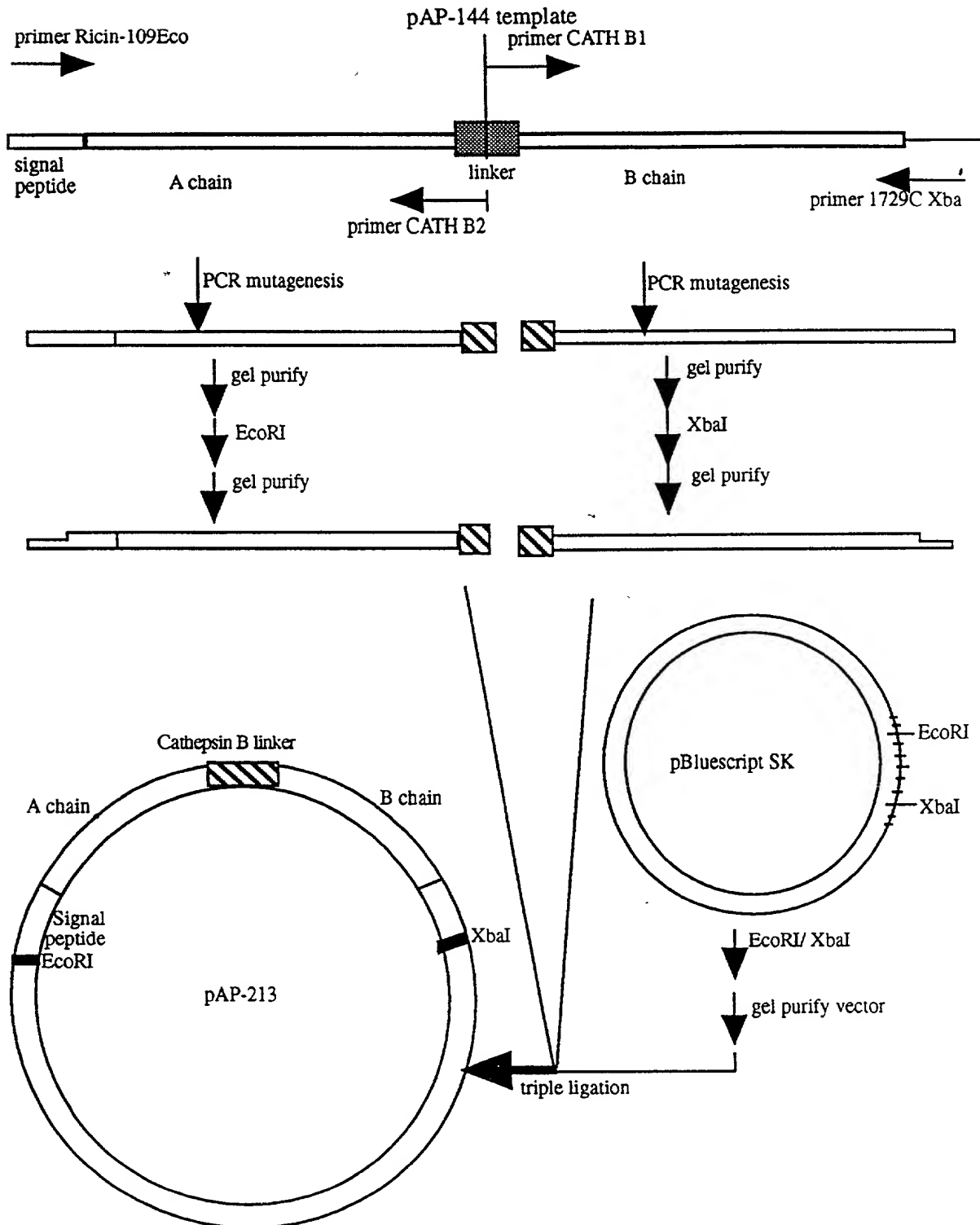
```
cgcgtcagcg ggtgttggcg ggtgtcgggg ctggcttaac tatgcggcat
cagagcagat tgtactgaga gtgcaccata tgcggtgtga aataccgcac
agatgcgtaa ggagaaaata ccgcatcagg cgccattcgc cattcaggct
gcgcaactgt tgggaagggc gatcggtgcg ggcctcttcg ctattacgcc
agctggcgaa agggggatgt gctgcaaggc gattaagttg ggtaacgcca
gggttttccc agtcacgacg ttgtaaaacg acggccagtg cc
```

//

0955151.041400

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**FIGURE 2A**





**FIGURE 2B**

WT preproinsulin linker

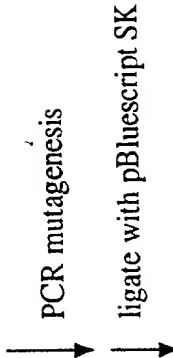
primer CATH-B1

5' - ATGGTGCCAAATTTTAAT-3'

TCTTTGCTTATAAGGCCAGTGGTGCCAAATTTTAAT  
AGAAACGAATATTCCGGTACCCACGGTTAAATTA

3' - TCTCGATTTAAGCAAGAAACTG-5'

primer CATH-B2

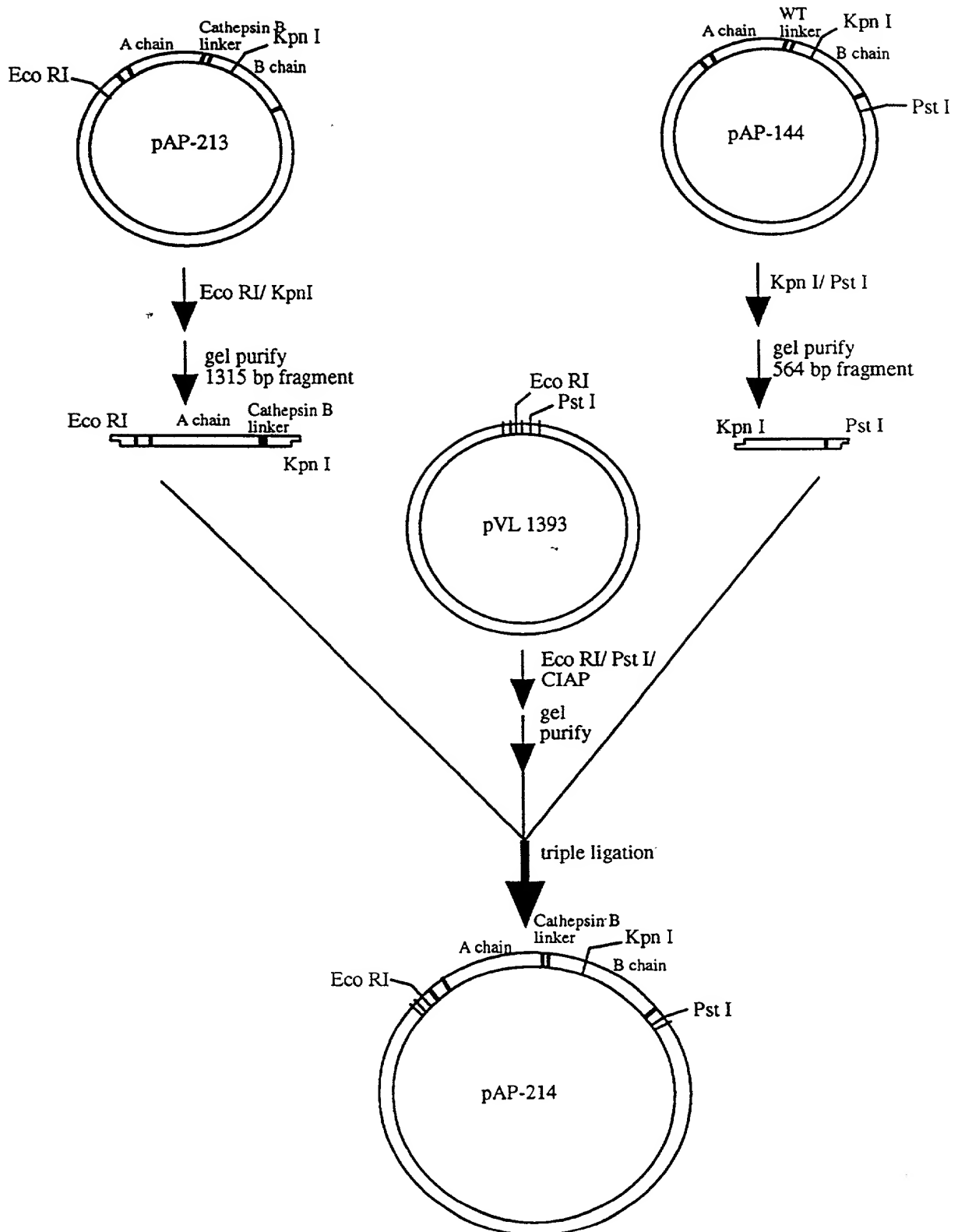


pAP 213 linker  
(Cathepsin-B variant)

TCTTTGCTTAAATCGAGAATGGTGCCAAATTTTAAT  
AGAAACGAATTTAGCTCTTACCACGGTTAAATTA

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**FIGURE 2C**



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## FIGURE 2D

10 20 30 40 50

1 GAATTCATGAAACCGGAGGAAATACTATTGTAATATGGATGTATGCAGT  
CTTAAGTACTTTGGCCCTCCTTTATGATAACATTATACCTACATACGTCA

51 GGCAACATGGCTTTGTTTGGATCCACCTCAGGGTGGTCTTTCACATTAG  
CCGTTGTACCGAAACAAAACCTAGGTGGAGTCCCACCAGAAAGTGTAACT

101 AGGATAACAACATATTCCCCAAACAATACCCAATTATAAACTTTACCACA  
TCCTATTGTTGTATAAGGGGTTGTTATGGGTAAATATTTGAAATGGTGT

151 GCGGGTGCCACTGTGCAAAGCTACACAACTTTATCAGAGCTGTTTCGCGG  
CGCCCACGGTGACACGTTTCGATGTGTTTGAAATAGTCTCGACAAGCGCC

201 TCGTTTAAACAAC TGGAGCTGATGTGAGACATGATATACCAGTGTTCGCAA  
AGCAAATTGTTGACCTCGACTACACTCTGTACTATATGGTCACAACGGTT

251 ACAGAGTTGGTTTGCCTATAAACCAACGGTTTATTTTAGTTGAACCTCTCA  
TGTCTCAACCAACGGATATTTGGTTGCCAAATAAAATCAACTTGAGAGT

301 AATCATGCAGAGCTTTCTGTTACATTAGCGCTGGATGTCACCAATGCATA  
TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT

351 TGTGGTTCGGCTACCGTGCTGGAAATAGCGCATATTTCTTTTCATCCTGACA  
ACACCAGCCGATGGCAGACCTTTATCGCGTATAAAGAAAGTAGGACTGT

401 ATCAGGAAGATGCAGAAGCAATCACTCATCTTTTCACTGATGTTCAAAAT  
TAGTCCTTCTACGTCTTCGTTAGTGAGTAGAAAAGTGA CTACAAGTTTTA

451 CGATATACATTTCGCTTTGGTGGTAATTATGATAGACTTGAACAACCTTGC  
GCTATATGTAAGCGGAAACCACCATTAATACTATCTGAACCTGTTGAACG

501 TGGTAATCTGAGAGAAAATATCGAGTTGGGAAATGGTCCACTAGAGGAGG  
ACCATTAGACTCTCTTTTATAGCTCAACCTTTACCAGGTGATCTCCTCC

551 CTATCTCAGCGCTTTATTATTACAGTACTGGTGGCACTCAGCTTCCAAC  
GATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA

601 CTGGCTCGTTTCCTTTATAATTTGCATCCAAATGATTTTCAAGCAGCAAG  
GACCGAGCAAGGAAATATTAAACGTAGGTTTACTAAAGTCTTCGTCGTTT

651 ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA  
TAAGGTTATATAACTCCCTCTTTACGCGTGCTCTTAATCCATGTTGGCCT

701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGGAGA  
CTAGACGTGGTCTAGGATCGCATTAATGTGAACTCTTATCAACCCCTCT

751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTTGCTAGTCCAAT  
GAAAGGTGACGTTAAGTTCTCAGATTGGTTCTCGGAAACGATCAGGTTA

801 TCAACTGCAAAGACGTAATGGTTCCAAATTCAGTGTGTACGATGTGAGTA  
AGTTGACGTTTCTGCATTACCAAGGTTTAAGTACACATGCTACACTCAT

851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGCACCTCCACCA  
ATAATTAGGGATAGTATCGAGAGTACCACATATCTACGCGTGAGGTGGT

901 TCGTCACAGTTTTCTTTTGCTTAAATCGAGAATGGTGCCAAATTTTAATGC  
AGCAGTGTCAAAAGAAACGAATTTAGCTCTTACCACGGTTTAAAATTACG

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## FIGURE 2D (CONT'D)

951 TGATGTTTGTATGGATCCTGAGCCCATAGTGCGTATCGTAGGTGAAATG  
ACTACAAACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTTAC

1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA  
CAGATACACAACACTACAATCCCTACCTTCTAAGGTGTTGCCTTTGCGTTAT

1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT  
GTCAACACCGGTACGTTTCAGATTATGTCTACGTTTAGTCGAGACCTGAAA

1101 GAAAAGAGACAATACTATTCGATCTAATGGAAAGTGTTAACTACTTACG  
CTTTTCTCTGTTATGATAAGCTAGATTACCTTTCACAAATTGATGAATGC

1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA  
CCATGTACGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT

1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC  
TGACTACGGTGGGCGACCGTTTATACCTTATTACCTTGGTAGTATTTAGG

1251 CAGATCTAGTCTAGTTTTAGCAGCGACATCAGGGAACAGTGGTACCACAC  
GTCTAGATCAGATCAAAATCGTCGCTGTAGTCCCTTGTCACCATGGTGTG

1301 TTACAGTGCAAACCAACATTTATGCCGTTAGTCAAGGTTGGCTTCCTACT  
AATGTCACGTTTGGTTGTAAATACGGCAATCAGTTCCAACCGAAGGATGA

1351 AATAATACACAACCTTTTTGTTACAACCATTGTTGGGCTATATGGTCTGTG  
TTATTATGTGTTGGAACAATGTTGGTAACAACCCGATATACCAGACAC

1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA  
GAACGTTTCGTTTATCACCTGTTTCATACCTATCTCCTGACATCGTCACTTT

1451 AGGCTGAACAACAGTGGGCTCTTTATGCAGATGGTTCAATACGTCCCTCAG  
TCCGACTTGTGTACCCCGAGAAATACGTCTACCAAGTTATGCAGGAGTC

1501 CAAAACCGAGATAATTGCCTTACAAGTGATTCTAATATACGGGAAACAGT  
GTTTTGGCTCTATTAACGGAATGTTCACTAAGATTATATGCCCTTTGTCA

1551 TGTTAAGATCCTCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT  
ACAATTCTAGGAGAGAAACACCGGGACGTAGGAGACCGGTTGCTACCTACA

1601 TCAAGAATGATGGAACCATTTTAAATTTGTATAGTGGATTGGTGTTAGAT  
AGTTCTTACTACCTTGGTAAAAATTTAAACATATCACCTAACCACAATCTA

1651 GTGAGGCGATCGGATCCGAGCCTTAAACAAATCATTCTTTACCCTCTCCA  
CACTCCGCTAGCCTAGGCTCGGAATTTGTTTAGTAAGAAATGGGAGAGGT

1701 TGGTGACCCAAACCAAATATGGTTACCATTATTTTGATAGACAGATTACT  
ACCACTGGGTTTGGTTTATACCAATGGTAATAAACTATCTGTCTAATGA

1751 CTCTTGCAGTGTGTGTCTCTGCCATGAAAAATAGATGGCTTAAATAAAAA  
GAGAACGTCACACACACAGGACGGTACTTTTATCTACCGAATTTATTTTTT

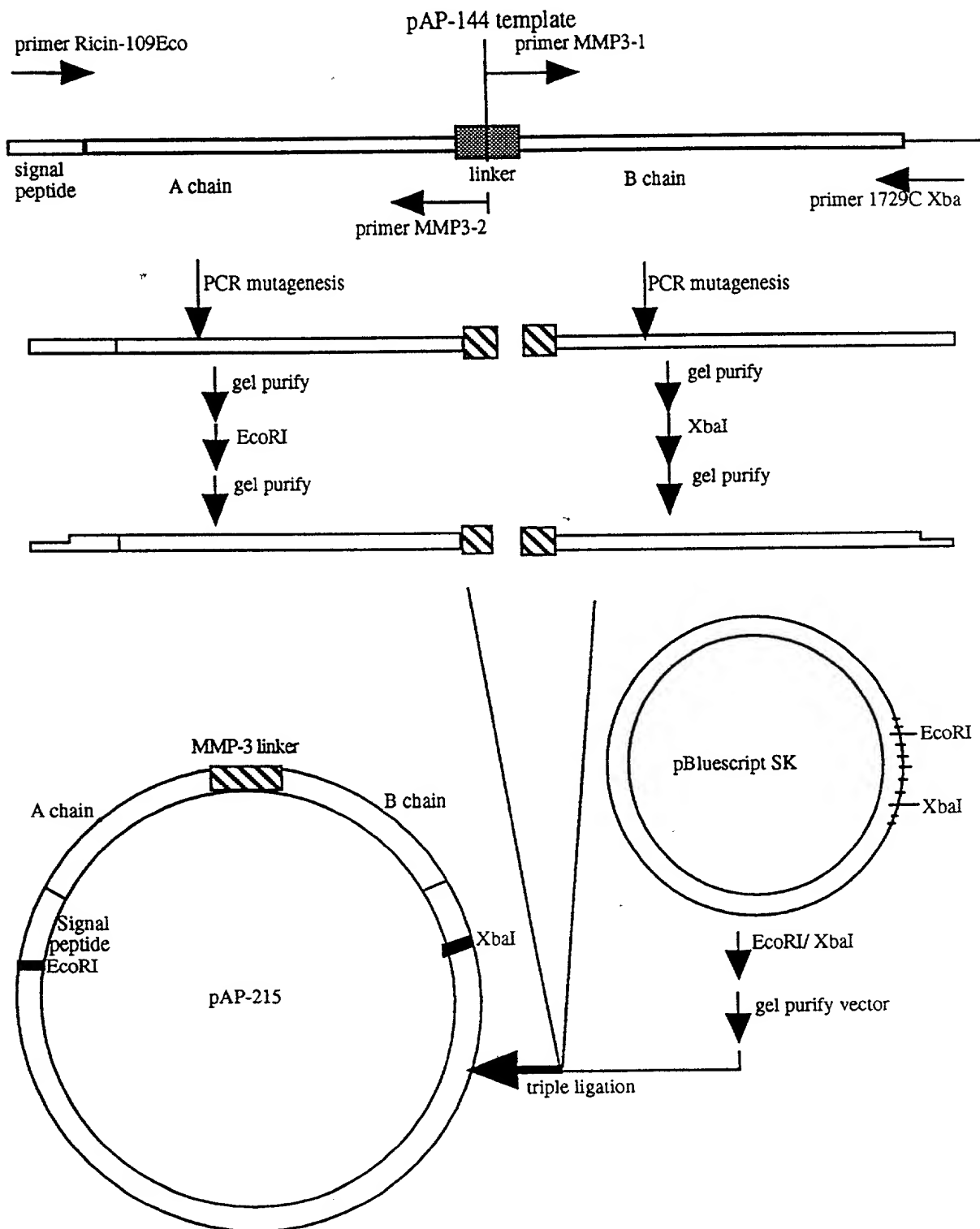
1801 GGACATTGTAAATTTTGTAACTGAAAGGACAGCAAGTTATATCGAATTCC  
CCTGTAACATTTAAACATTGACTTTCCTGTCGTTCAATATAGCTTAAGG

1851 TGCAG  
ACGTC

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**FIGURE 3A**



## FIGURE 3B

### WT preprorin linker

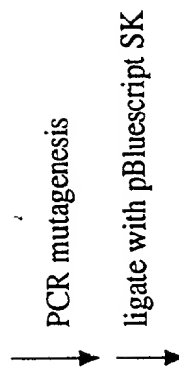
primer MMP3-1

5' - TTTTGGACTTATGAATGCTGATGTTTGT -3'

TTTTCGCTTATAAGGCCAGTGGTGCCCAATTTTAAT  
AGAAACGAATATCCGGTCACCCAGGTTTAAATTA

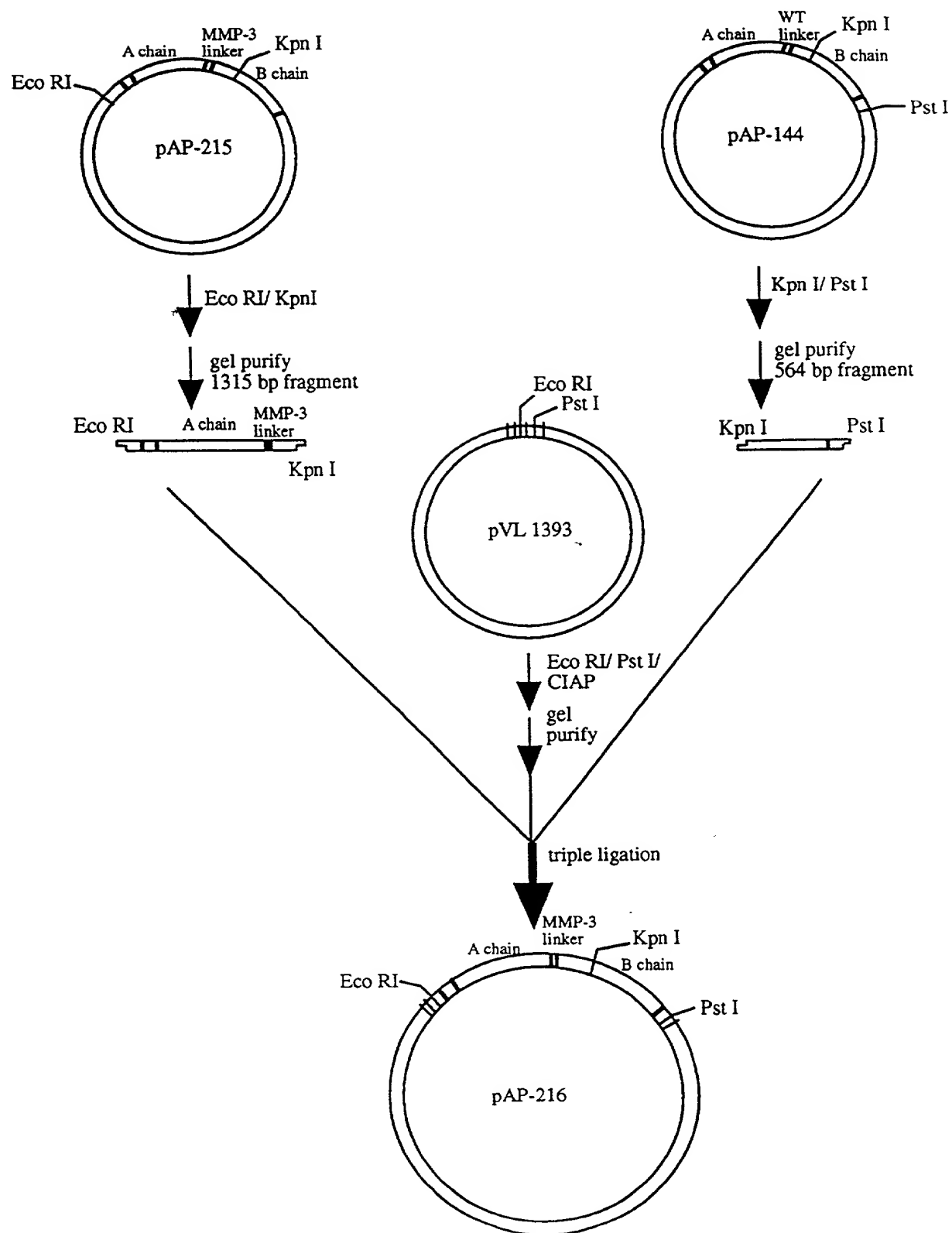
3' - GGTAGCAGTGTCAAAGCAGGCTTCGGTTCGTT -5'

primer MMP3-2



pAP 215 linker  
(MMP-3 variant)

CGTCCGAAGCCACAGCAATTTTGGACTTATGAAT  
GCAGGCTTCGGTTCGTTAAAAAACCTGAATACTTA

**FIGURE 3C**

**FIGURE 3D**

10 20 30 40 50  
 1 GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT  
 CTTAAGTACTTTGGCCCTCCTTTATGATAACATTATACCTACATACGTCA  
 51 GGCAACATGGCTTTGTTTTGGATCCACCTCAGGGTGGTCTTTCACATTAG  
 CCGTTGTACCGAAACAAAACCTAGGTGGAGTCCCACCAGAAAGTGTAATC  
 101 AGGATAACAACATATTCCCCAAACAATACCCAATTATAAACTTTACCACA  
 TCCTATTGTTGTATAAGGGGTTTGTATGGGTTAATATTTGAAATGGTGT  
 151 GCGGGTGCCACTGTGCAAAGCTACACAAACTTTATCAGAGCTGTTTCGCGG  
 CGCCACGGTGACACGTTTCGATGTGTTTGAAATAGTCTCGACAAGCGCC  
 201 TCGTTTAACAACCTGGAGCTGATGTGAGACATGATATACCAGTGTGCGCA  
 AGCAAATTGTTGACCTCGACTACACTCTGTACTATATGGTCACAACGGTT  
 251 ACAGAGTTGGTTTGCCTATAAACCAACGGTTTATTTTAGTTGAACTCTCA  
 TGTCTCAACCAAACGGATATTTGGTTGCCAAATAAAATCAACTTGAGAGT  
 301 AATCATGCAGAGCTTTCTGTTACATTAGCGCTGGATGTCACCAATGCATA  
 TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT  
 351 TGTGGTCGGCTACCGTGCTGGAATAGCGCATATTTCTTTCATCCTGACA  
 ACACCAGCCGATGGCACGACCTTTATCGCGTATAAAGAAAGTAGGACTGT  
 401 ATCAGGAAGATGCAGAAGCAATCACTCATCTTTTTEACTGATGTTCAAAAT  
 TAGTCCTTCTACGTCTTCGTTAGTGAGTAGAAAAGTGACTACAAGTTTTA  
 451 CGATATACATTTCGCCTTTGGTGGTAATTATGATAGACTTGAACAACTTGC  
 GCTATATGTAAGCGGAAACCACCATTAACTACTATCTGAACCTGTTGAACG  
 501 TGGTAATCTGAGAGAAAATATCGAGTTGGGAAATGGTCCACTAGAGGAGG  
 ACCATTAGACTCTCTTTTATAGCTCAACCCTTTACCAGGTGATCTCCTCC  
 551 CTATCTCAGCGCTTTATTATTACAGTACTGGTGGCACTCAGCTTCCAAC  
 GATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA  
 601 CTGGCTCGTTCCTTTATAATTTGCATCCAAATGATTTTCAAGCAGCAAG  
 GACCGAGCAAGGAAATATTAAACGTAGGTTTACTAAAGTCTTCGTCGTTT  
 651 ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA  
 TAAGGTTATATAACTCCCTCTTTACGCGTGCTCTTAATCCATGTTGGCCT  
 701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGGAGA  
 CTAGACGTGGTCTAGGATCGCATTAAATGTGAACTCTTATCAACCCCTCT  
 751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTTGCTAGTCCAAT  
 GAAAGGTGACGTTAAGTTCTCAGATTGGTTCTCGGAAACGATCAGGTTA  
 801 TCAACTGCAAAGACGTAATGGTTCCAAATTCAGTGTGTACGATGTGAGTA  
 AGTTGACGTTTCTGCATTACCAAGGTTTAAAGTCACACATGCTACACTCAT  
 851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGCACCTCCACCA  
 ATAATTAGGGATAGTATCGAGAGTACCACATATCTACGCGTGAGGTGGT  
 901 TCGTCACAGTTTCGTCCGAAGCCACAGCAATTTTTTGGACTTATGAATGC  
 AGCAGTGTCAAAGCAGGCTTCGGTGTCTGTTAAAAAACCTGAATACTTACG  
 951 TGATGTTTGTATGGATCCTGAGCCCATAGTGCGTATCGTAGGTGCGAAATG



**FIGURE 3D (CONT'D)**

ACTACAAACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTTAC

1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA  
CAGATACACAACACTACAATCCCTACCTTCTAAGGTGTTGCCTTTGCGTTAT

1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT  
GTCAACACCGGTACGTTTCAGATTATGTCTACGTTTAGTCGAGACCTGAAA

1101 GAAAAGAGACAATACTATTTCGATCTAATGGAAAGTGTTTAACTACTTACG  
CTTTTCTCTGTTATGATAAGCTAGATTACCTTTCACAAATTGATGAATGC

1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA  
CCATGTCAGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT

1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC  
TGACTACGGTGGGCGACCGTTTATACCTATTACCTTGGTAGTATTTAGG

1251 CAGATCTAGTCTAGTTTTAGCAGCGACATCAGGGAACAGTGGTACCACAC  
GTCTAGATCAGATCAAAATCGTCGCTGTAGTCCCTTGTCAACCATGGTGTG

1301 TTACAGTGCAAACCAACATTTATGCCGTTAGTCAAGGTTGGCTTCCTACT  
AATGTCACGTTTGGTTGTAAATACGGCAATCAGTTCCAACCGAAGGATGA

1351 AATAATACACAACCTTTTGTGTACAACCATTGTTGGGCTATATGGTCTGTG  
TTATTATGTGTTGGAAAACAATGTTGGTAACAACCCGATATACCAGACAC

1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA  
GAACGTTTCGTTTATCACCTGTTTCATACCTATCTCCTGACATCGTCACTTT

1451 AGGCTGAACAACAGTGGGCTCTTTATGCAGATGGTTCAATACGTCCTCAG  
TCCGACTTGTGTGCACCCGAGAAATACGTCTACCAAGTTATGCAGGAGTC

1501 CAAAACCGAGATAATTGCCTTACAAGTGATTCTAATATACGGGAAACAGT  
GTTTTGGCTCTATTAACGGAATGTTCACTAAGATTATATGCCCTTTGTCA

1551 TGTTAAGATCCTCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT  
ACAATTCTAGGAGAGAAACACCGGGACGTAGGAGACCGGTTGCTACCTACA

1601 TCAAGAATGATGGAACCATTTTAAATTTGTATAGTGGATTGGTGTTAGAT  
AGTTCTTACTACCTTGGTAAAAATTTAAACATATCACCTAACCACAATCTA

1651 GTGAGGCGATCGGATCCGAGCCTTAAACAAATCATTCTTTACCCCTCTCCA  
CACTCCGCTAGCCTAGGCTCGGAATTTGTTTAGTAAGAAATGGGAGAGGT

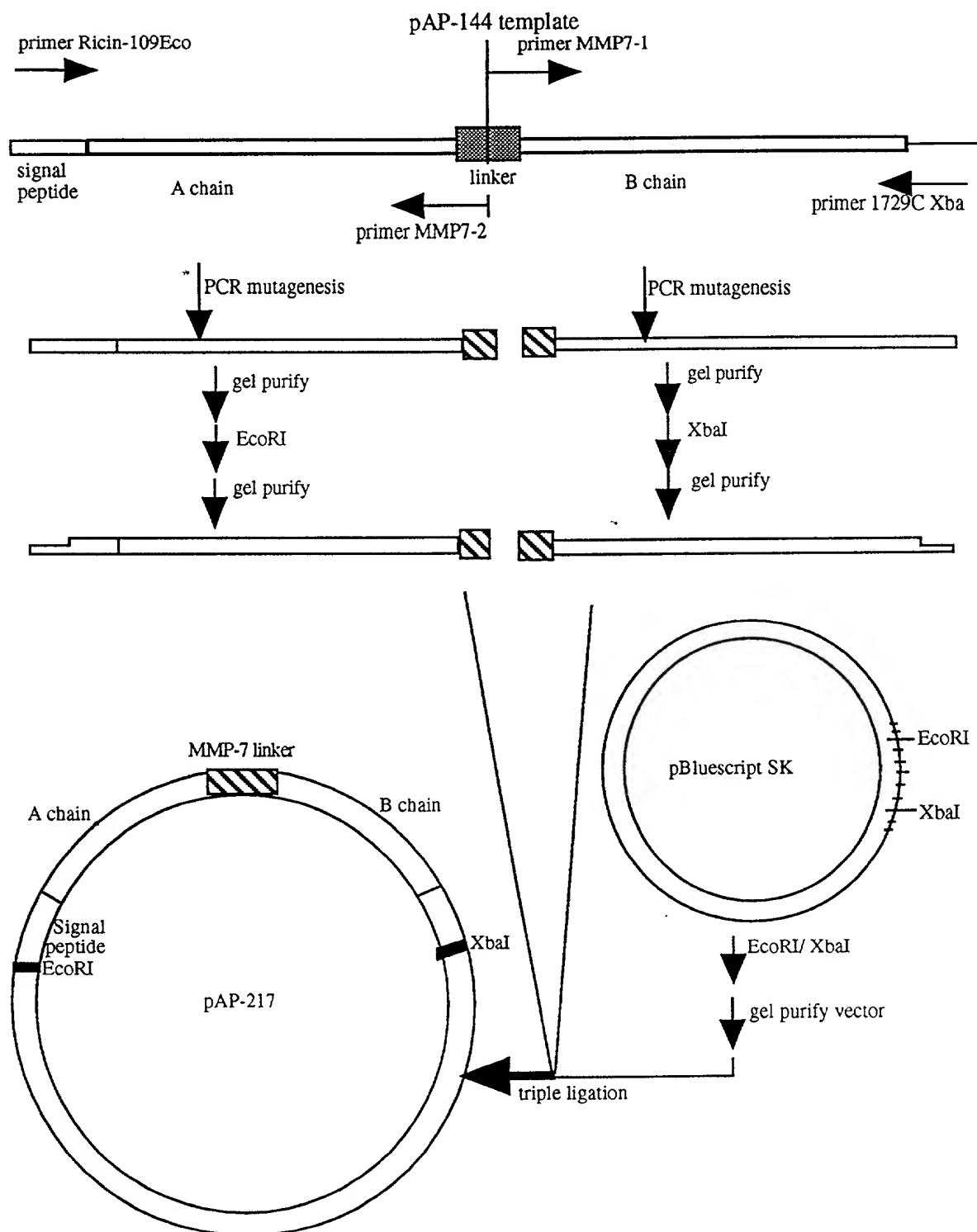
1701 TGGTGACCCAAACCAAATATGGTTACCATTATTTTGATAGACAGATTACT  
ACCACTGGGTTTGGTTTATACCAATGGTAATAAACTATCTGTCTAATGA

1751 CTCTTGCACTGTGTGTGTCTCTGCCATGAAAATAGATGGCTTAAATAAAAA  
GAGAACGTCACACACACAGGACGGTACTTTTATCTACCGAATTTATTTTT

1801 GGACATTGTAAATTTTGTAACTGAAAGGACAGCAAGTTATATCGAATTCC  
CCTGTAACATTTAAACATTGACTTTCCTGTGCTTCAATATAGCTTAAGG

1851 TGCAG  
ACGTC

FIGURE 4A



## FIGURE 4B

WT preproincin linker

primer MMP7-1

5' - TTGTGGCGAAGTTTAAATGCTGATGTT-3'

TCCTTGCTTATAAGGCCAGTGGTGCCAAATTTTAAT  
AGAAACGAAATATCCGGTCACCCACGGTTTAAATTA

3' - AGTGTCAAAAGAACGACGGTGACCGT-5'

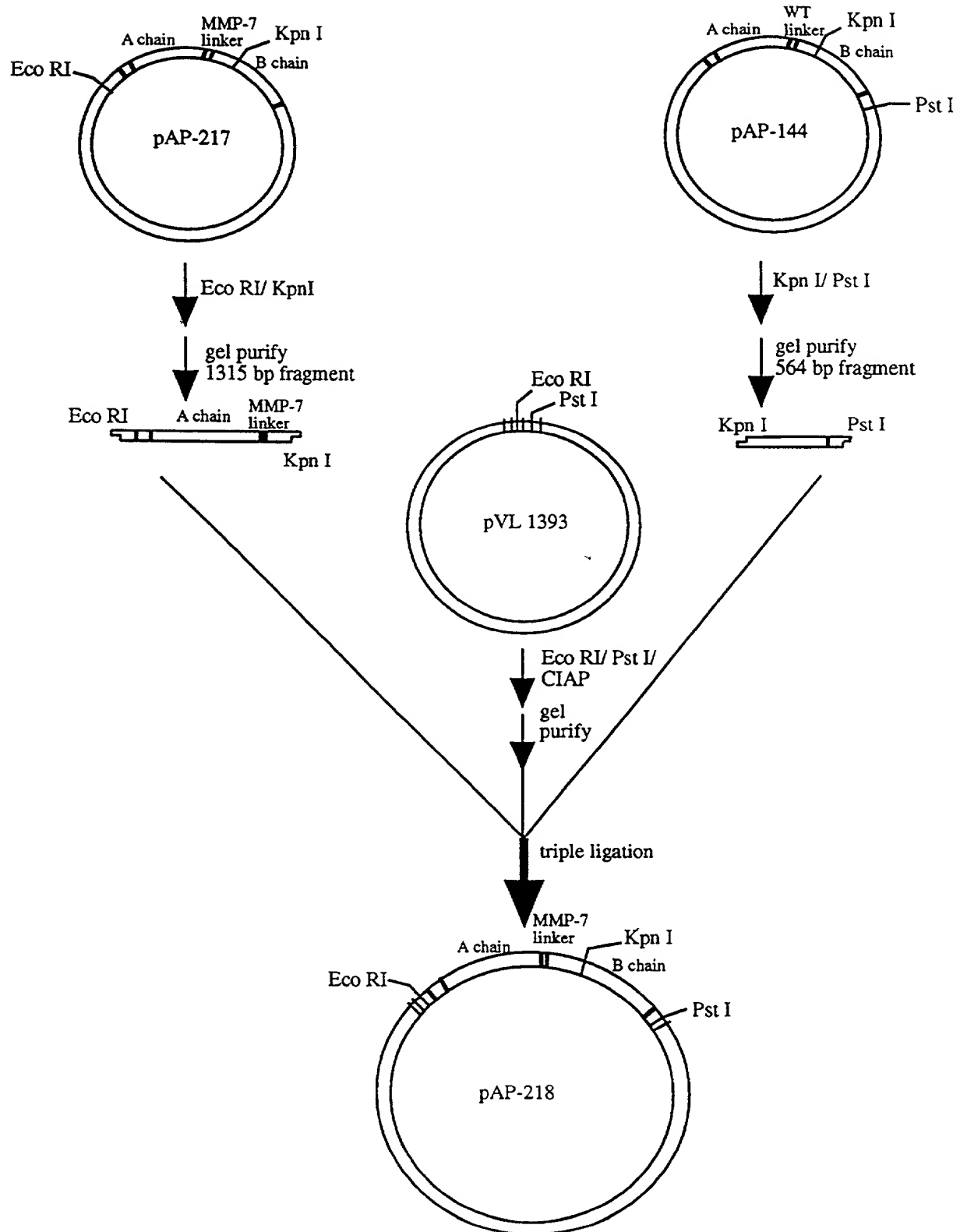
primer MMP7-2

PCR mutagenesis

ligate with pBluescript SK

pAP 217 linker  
(MMP-7 variant)

TCCTTGCGTCCACTGGCATTTGTGGCGAAGTTTAAAT  
AGAAACGCAGGTGACCGTAACACCGCTTCAAAATTA

**FIGURE 4C**

**FIGURE 4D**

10 20 30 40 50

1 GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT  
CTTAAGTACTTTGGCCCTCCTTTATGATAACATTATACCTACATACGTCA

51 GGCAACATGGCTTTGTTTTGGATCCACCTCAGGGTGGTCTTTCACATTAG  
CCGTTGTACCGAAACAAAACCTAGGTGGAGTCCCACCAGAAAGTGAATC

101 AGGATAACAACATATTCCCCAAACAATACCCAATTATAAACTTTACCACA  
TCCTATTGTTGTATAAGGGGTTTGTATGGGTAAATATTTGAAATGGTGT

151 GCGGGTGCCACTGTGCAAAGCTACACAACTTTATCAGAGCTGTTGCGGG  
CGCCACGGTGACACGTTTCGATGTGTTTGAAATAGTCTCGACAAGCGCC

201 TCGTTTAACAACCTGGAGCTGATGTGAGACATGATATACAGTGTGCCAA  
AGCAAATTGTTGACCTCGACTACACTCTGTACTATATGGTCACAACGGTT

251 ACAGAGTTGGTTTGCCTATAAACCAACGGTTTATTTTAGTTGAACTCTCA  
TGTCTCAACCAAACGGATATTTGGTTGCCAAATAAAATCAACTTGAGAGT

301 AATCATGCAGAGCTTCTGTACATTAGCGCTGGATGTCACCAATGCATA  
TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT

351 TGTGGTCGGCTACCGTGCTGGAAATAGCGCATATTTCTTTCATCCTGACA  
ACACCAGCCGATGGCACGACCTTTATCGCGTATAAAGAAAGTAGGACTGT

401 ATCAGGAAGATGCAGAAGCAATCACTCATCTTTTCACTGATGTTCAAAT  
TAGTCCTTCTACGTCTTCGTTAGTGAGTAGAAAAGTGACTACAAGTTTTA

451 CGATATACATTTCGCTTTGGTGGTAATTATGATAGACTTGAACAACTTGC  
GCTATATGTAAGCGGAAACCACCATTAATACTATCTGAACCTGTTGAACG

501 TGGTAATCTGAGAGAAAATATCGAGTTGGGAAATGGTCCACTAGAGGAGG  
ACCATTAGACTCTCTTTTATAGCTCAACCCTTTACCAGGTGATCTCCTCC

551 CTATCTCAGCGCTTTATTATTACAGTACTGGTGGCACTCAGCTTCCAAC  
GATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA

601 CTGGCTCGTTCCTTTATAATTTGCATCCAAATGATTTCAGAAGCAGCAAG  
GACCGAGCAAGGAAATATTAAACGTAGGTTTACTAAAGTCTTCGTCGTTT

651 ATTCCAATATATTGAGGGAGAAATGCGCACGAGAAATTAGGTACAACCGGA  
TAAGGTTATATAACTCCCTCTTTACGCGTGCTCTTAATCCATGTTGGCCT

701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGGAGA  
CTAGACGTGGTCTAGGATCGCATTAATGTGAACCTTATCAACCCCTCT

751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTTGCTAGTCCAAT  
GAAAGGTGACGTTAAGTTCTCAGATTGGTTCCCTCGGAAACGATCAGGTTA

801 TCAACTGCAAAGACGTAATGGTTCCAAATTCAGTGTGTACGATGTGAGTA  
AGTTGACGTTTCTGCATTACCAAGGTTTAAGTCAACATGCTACACTCAT

851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGCACCTCCACCA  
ATAATTAGGGATAGTATCGAGAGTACCACATATCTACGCGTGAGGTGGT

901 TCGTCACAGTTTTCTTTGCGTCCACTGGCATTGTGGCGAAGTTTTAATGC  
AGCAGTGTCAAAGAAACGCAGGTGACCGTAACACCGCTTCAAATACG

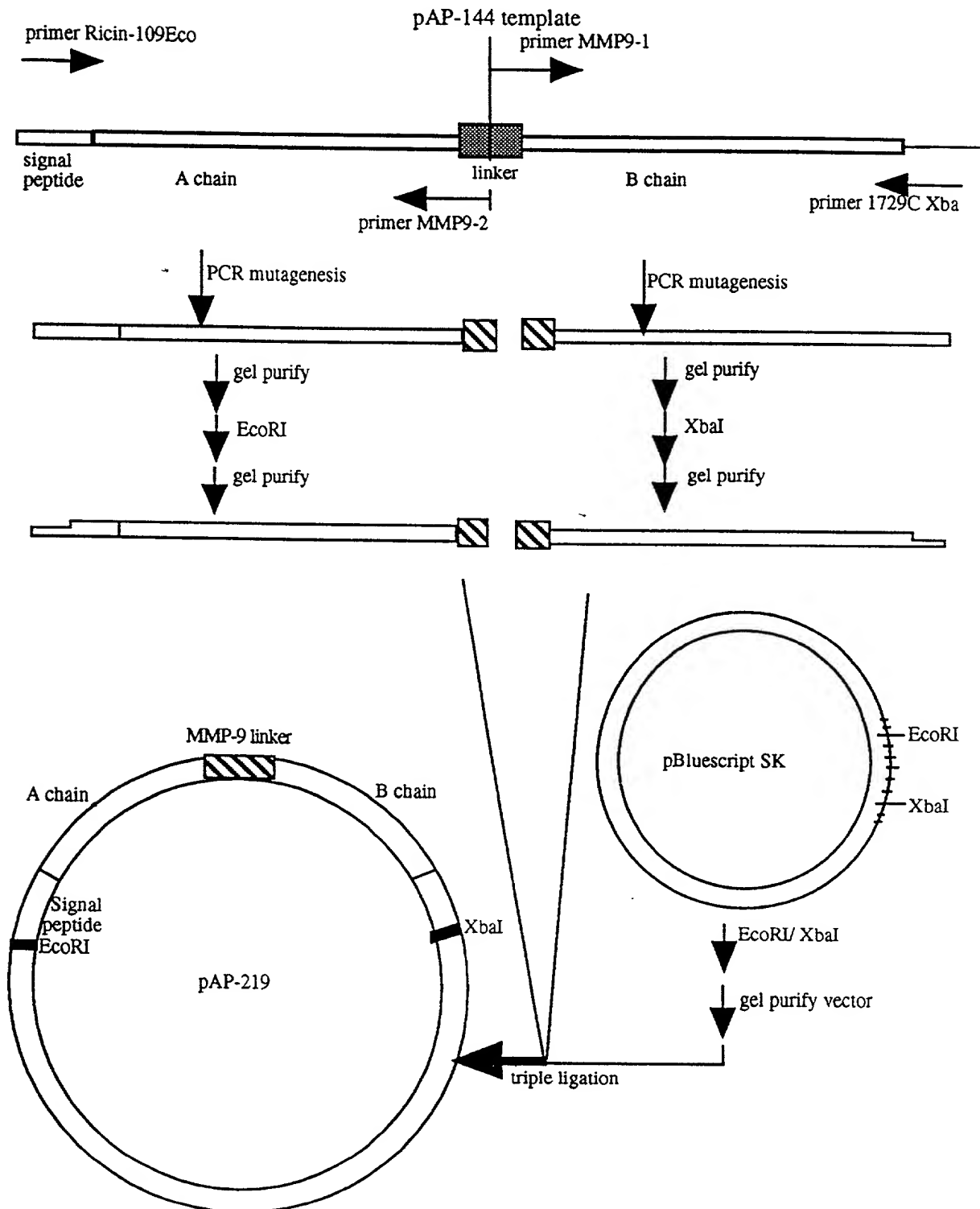
951 TGATGTTTGTATGGATCCTGAGCCCATAGTGCATCGTAGGTGCAATG

**FIGURE 4D (CONT'D)**

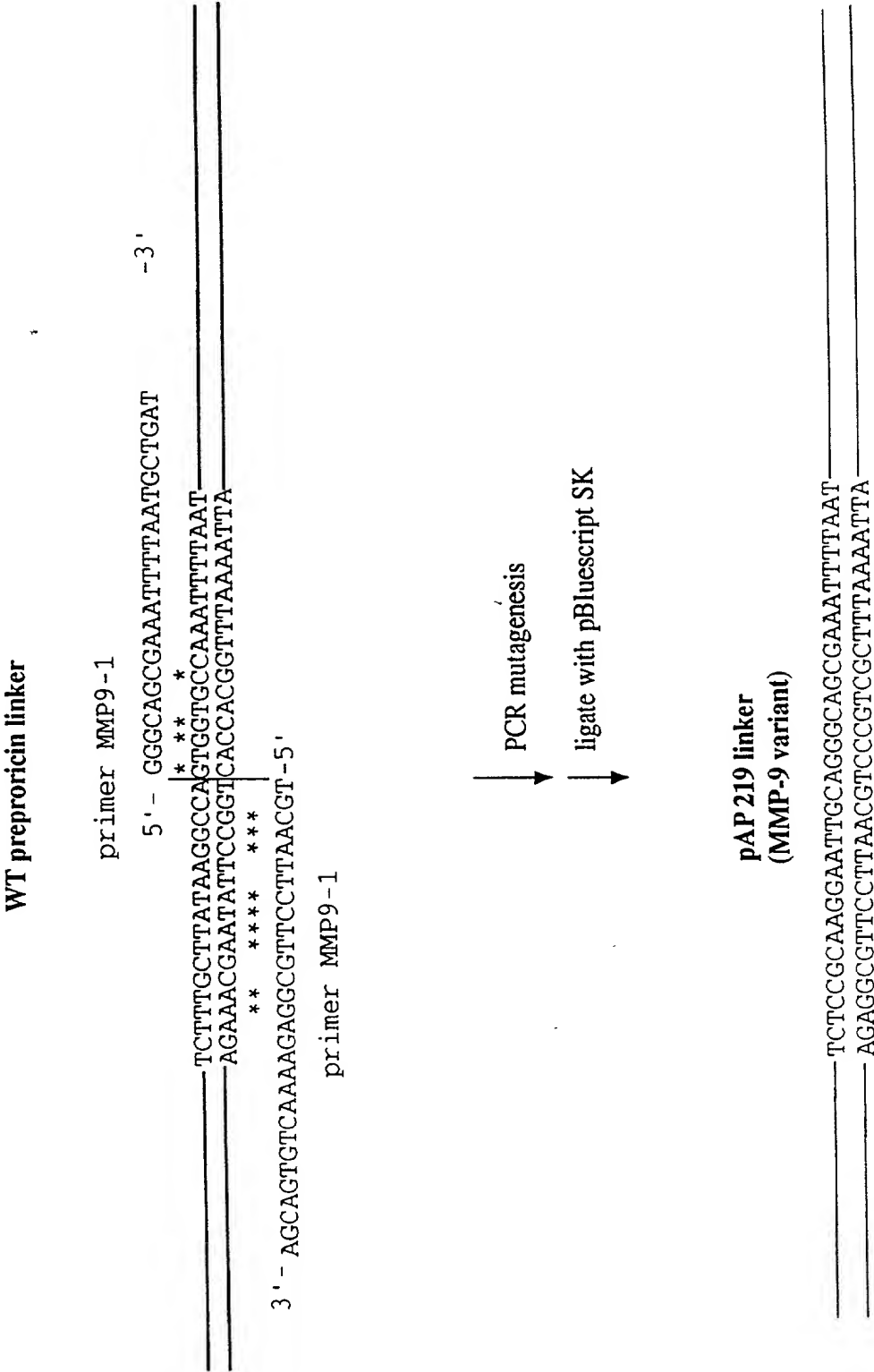
ACTACAAACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTTAC  
 1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA  
 CAGATACACAACCTACAATCCCTACCTTCTAAGGTGTTGCCTTTGCGTTAT  
 1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT  
 GTCAACACCGGTACGTTTACGATTATGTCTACGTTTAGTCGAGACCTGAAA  
 1101 GAAAAGAGACAATACTATTTCGATCTAATGGAAAGTGTTAACTACTTACG  
 CTTTCTCTGTTATGATAAGCTAGATTACCTTTCACAAATTGATGAATGC  
 1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA  
 CCATGTTCAGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT  
 1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC  
 TGACTACGGTGGGCGACCGTTTATACCTTATTACCTTGGTAGTATTTAGG  
 1251 CAGATCTAGTCTAGTTTTAGCAGCGACATCAGGGAACAGTGGTACCACAC  
 GTCTAGATCAGATCAAAATCGTCGCTGTAGTCCCTTGTCAACCATGGTGTG  
 1301 TTACAGTGCAAACCAACATTTATGCCGTTAGTCAAGGTTGGCTTCCTACT  
 AATGTCACGTTTGGTTGTAAATACGGCAATCAGTTCCAACCGAAGGATGA  
 1351 AATAATACACAACCTTTTGTACAAACCATTTGTTGGGCTATATGGTCTGTG  
 TTATTATGTGTTGGAAAACAATGTTGGTAACAACCCGATATACCAGACAC  
 1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA  
 GAACGTTTCGTTTATCACCTGTTTCATACCTATCTCCTGACATCGTCACTTT  
 1451 AGGCTGAACAACAGTGGGCTCTTTATGCAGATGGTTCAATACGTCCTCAG  
 TCCGACTTGTGTGTCACCCGAGAAATACGTCTACCAAGTTATGCAGGAGTC  
 1501 CAAAACCGAGATAATTGCCTTACAAGTGATTCTAATATACGGGAAACAGT  
 GTTTTGGCTCTATTAACGGAATGTTCACTAAGATTATATGCCCTTTGTCA  
 1551 TGTTAAGATCCTCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT  
 ACAATTCTAGGAGAGAAACACCGGGACGTAGGAGACCGGTTGCTACCTACA  
 1601 TCAAGAATGATGGAACCATTTTAAATTTGTATAGTGGATTGGTGTAGAT  
 AGTTCTTACTACCTTGGTAAAATTTAAACATATCACCTAACCAATCTA  
 1651 GTGAGGCGATCGGATCCGAGCCTTAAACAAATCATTCTTTACCCTCTCCA  
 CACTCCGCTAGCCTAGGCTCGGAATTTGTTTAGTAAGAAATGGGAGAGGT  
 1701 TGGTGACCCAAACCAATATGGTTACCATTATTTTGATAGACAGATTACT  
 ACCACTGGGTTTGGTTTATACCAATGGTAATAAACTATCTGTCTAATGA  
 1751 CTCTTGCAAGTGTGTGTCTGTCCTGCCATGAAAATAGATGGCTTAAATAAAAA  
 GAGAACGTCACACACACAGGACGGTACTTTTATCTACCGAATTTATTTTT  
 1801 GGACATTGTAAATTTTGTAACTGAAAGGACAGCAAGTTATATCGAATTCC  
 CCTGTAACATTTAAACATTGACTTTTCTGTGTCGTTCAATATAGCTTAAGG  
 1851 TGCAG  
 ACGTC

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**FIGURE 5A**



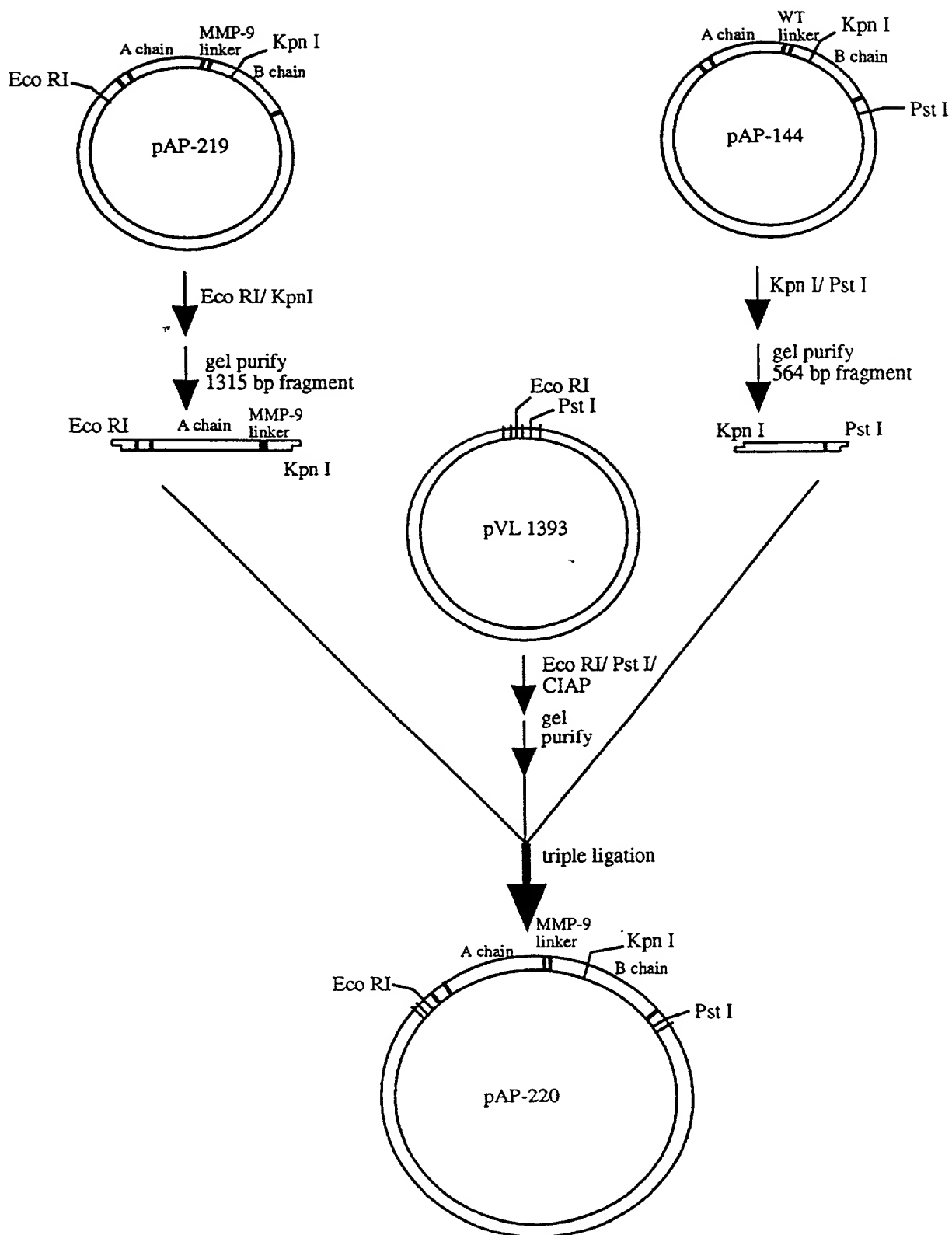
**FIGURE 5B**





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**FIGURE 5C**

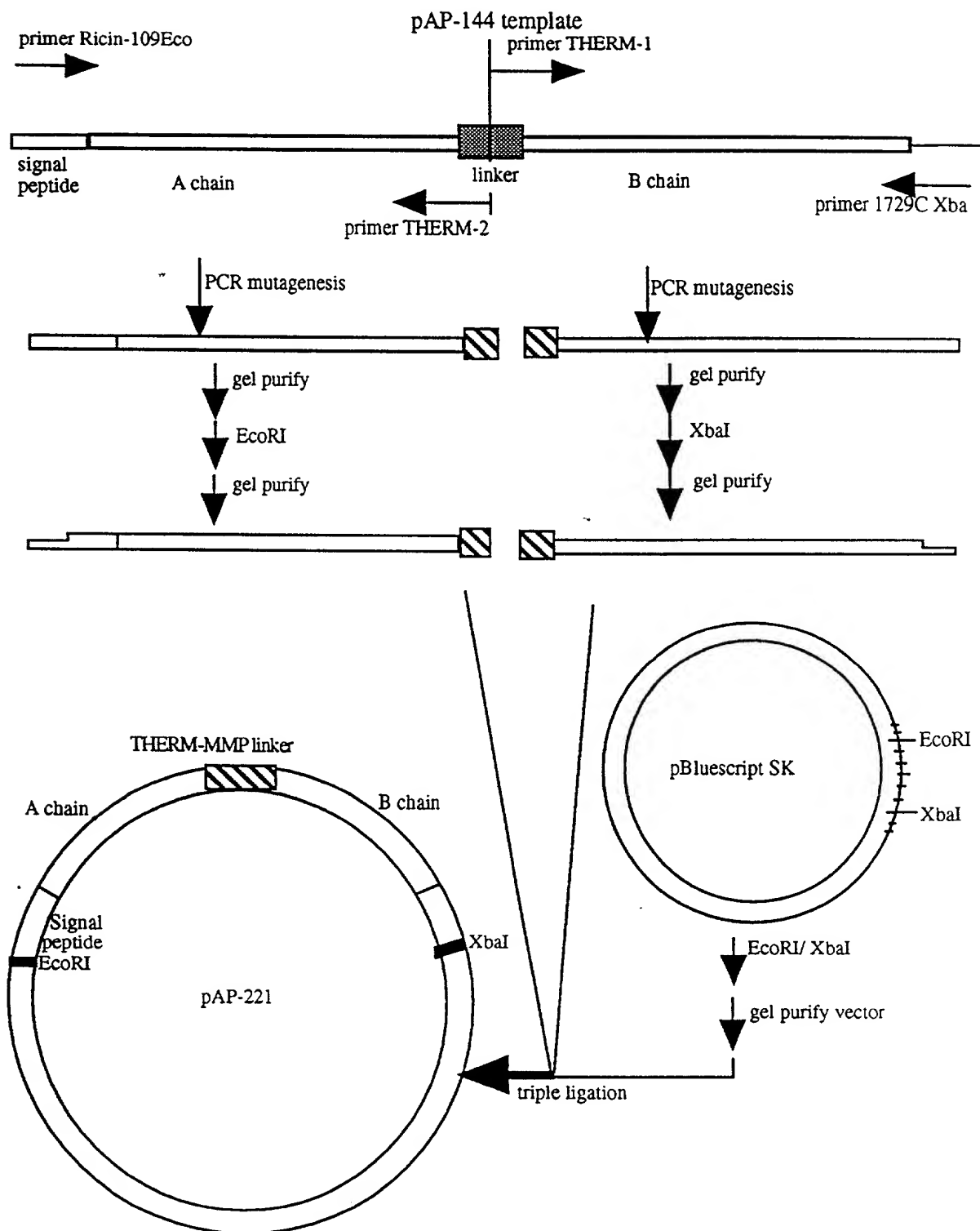


**FIGURE 5D**

10                      20                      30                      40                      50  
 1 GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT  
 CTTAAGTACTTTGGCCCTCCTTTATGATAACATTATACCTACATACGTCA  
 51 GGCAACATGGCTTTGTTTTGGATCCACCTCAGGGTGGTCTTTCACATTAG  
 CCGTTGTACCGAAACAAACCTAGGTGGAGTCCCACCAGAAAGTGTAAATC  
 101 AGGATAACAACATATTCCCCAAACAATACCCAATTATAAACTTTACCACA  
 TCCTATTGTTGTATAAGGGGTTTGTATGGGTAAATATTTGAAATGGTGT  
 151 GCGGGTGCCACTGTGCAAGCTACACAACTTTATCAGAGCTGTTGCGCG  
 CGCCACGGTGACACGTTTCGATGTGTTGAAATAGTCTCGACAAGCGCC  
 201 TCGTTTAACAACCTGGAGCTGATGTGAGACATGATATACCAGTGTGCCAA  
 AGCAAATTGTTGACCTCGACTACACTCTGTACTATATGGTCACAACGGTT  
 251 ACAGAGTTGGTTTGCCTATAAACCAACGGTTTATTTTAGTTGAACTCTCA  
 TGTCTCAACCAACGGATATTTGGTTGCCAAATAAAATCAACTTGAGAGT  
 301 AATCATGCAGAGCTTCTGTACATTAGCGCTGGATGTCACCAATGCATA  
 TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT  
 351 TGTGGTTCGGCTACCGTGCTGGAAATAGCGCATATTTCTTTTCATCCTGACA  
 ACACCAGCCGATGGCAGCACCCTTATCGCGTATAAAGAAAGTAGGACTGT  
 401 ATCAGGAAGATGCAGAAAGCAATCACTCATCTTTTCACTGATGTTCAAAAT  
 TAGTCCTTCTACGTCTTCGTTAGTGAGTAGAAAAGTGACTACAAGTTTTA  
 451 CGATATACATTTCGCCCTTTGGTGGTAATTATGATAGACTTGAACAACCTTGC  
 GCTATATGTAAGCGGAAACCACCATTAATACTATCTGAACCTGTTGAACG  
 501 TGGTAATCTGAGAGAAAATATCGAGTTGGGAAATGGTCCACTAGAGGAGG  
 ACCATTAGACTCTCTTTTATAGCTCAACCCTTTACCAGGTGATCTCCTCC  
 551 CTATCTCAGCGCTTTATTATTACAGTACTGGTGGCACTCAGCTTCCAAC  
 GATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA  
 601 CTGGCTCGTTCCCTTTATAAATTGTCATCCAAATGATTTTCAAGCAGCAAG  
 GACCGAGCAAGGAAATATTAAACGTAGGTTTACTAAAGTCTTCGTCGTTT  
 651 ATTCCAATATATTGAGGGAGAAATGCGCACGAGAAATTAGGTACAACCGGA  
 TAAGGTTATATAACTCCCTCTTTACGCGTGCTCTTAATCCATGTTGGCCT  
 701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGGAGA  
 CTAGACGTGGTCTAGGATCGCATTAATGTGAACCTTATCAACCCCTCT  
 751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTTGCTAGTCCAAT  
 GAAAGGTGACGTTAAGTTCTCAGATTGGTTCCTCGGAAACGATCAGGTTA  
 801 TCAACTGCAAAGACGTAATGGTTCCAAATTCAGTGTGTACGATGTGAGTA  
 AGTTGACGTTTCTGCATTACCAAGGTTTAAGTCACACATGCTACACTCAT  
 851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGCACCTCCACCA  
 ATAATTAGGGATAGTATCGAGAGTACCACATATCTACGCGTGGAGGTGGT  
 901 TCGTCACAGTTTTCTCCGCAAGGAATTGCAGGGCAGCGAAATTTTAATGC  
 AGCAGTGTCAAAAGAGGCGTTCCCTTAACGTCCCGTCGCTTTAAATTACG

**FIGURE 5D (CONT'D)**

951 TGATGTTTGTATGGATCCTGAGCCCATAGTGCGTATCGTAGGTCGAAATG  
 ACTACAAACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTTAC  
 1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA  
 CAGATACACAACACTACAATCCCTACCTTCTAAGGTGTTGCCTTTGCGTTAT  
 1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT  
 GTCAACACCGGTACGTTTACAGATTATGTCTACGTTTAGTCGAGACCTGAAA  
 1101 GAAAAGAGACAATACTATTCGATCTAATGGAAAGTGTTTAACTACTTACG  
 CTTTTCTCTGTTATGATAAGCTAGATTACCTTTCACAAATTGATGAATGC  
 1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA  
 CCATGTCAGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT  
 1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC  
 TGACTACGGTGGGCGACCGTTTATACCCTATTACCTTGGTAGTATTTAGG  
 1251 CAGATCTAGTCTAGTTTTAGCAGCGACATCAGGGAACAGTGGTACCACAC  
 GTCTAGATCAGATCAAAATCGTCGCTGTAGTCCCTTGTCAACCATGGTGTG  
 1301 TTACAGTGCAAACCAACATTTATGCCGTTAGTCAAGGTTGGCTTCCTACT  
 AATGTCACGTTTGGTTGTAAATACGGCAATCAGTTCCAACCGAAGGATGA  
 1351 AATAATACACAACCTTTTGTTACAACCATTGTTGGGCTATATGGTCTGTG  
 TTATTATGTGTTGGAAAACAATGTTGGTAACAACCCGATATACCAGACAC  
 1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA  
 GAACGTTTCGTTTATCACCTGTTTATACCTATCTCCTGACATCGTCACTTT  
 1451 AGGCTGAACAACAGTGGGCTCTTTATGCAGATGGTTCAATACGTCCTCAG  
 TCCGACTTGTGTCAACCGAGAAATACGTCTACCAAGTTATGCAGGAGTC  
 1501 CAAAACCGAGATAATTGCCTTACAAGTGATTCTAATATACGGGAAACAGT  
 GTTTTGGCTCTATTAAACGGAATGTTCACTAAGATTATATGCCCTTTGTCA  
 1551 TGTTAAGATCCTCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT  
 ACAATTCTAGGAGAGAAACACCGGGACGTAGGAGACCGGTTGCTACCTACA  
 1601 TCAAGAAATGATGGAACCATTTTAAATTTGTATAGTGGATTGGTGTTAGAT  
 AGTTCCTTACTACCTTGGTAAAATTTAAACATATCACCTAACCACAATCTA  
 1651 GTGAGGCGATCGGATCCGAGCCTTAAACAAATCATTCTTTACCCTCTCCA  
 CACTCCGCTAGCCTAGGCTCGGAATTTGTTTAGTAAGAAATGGGAGAGGT  
 1701 TGGTGACCCAAACCAAATATGGTTACCATTATTTTGATAGACAGATTACT  
 ACCACTGGGTTTGGTTTATACCAATGGTAATAAACTATCTGTCTAATGA  
 1751 CTCTTGCAAGTGTGTGTGCTGCGCATGAAAATAGATGGCTTAAATAAAAA  
 GAGAACGTCACACACACAGGACGGTACTTTTATCTACCGAATTTATTTTT  
 1801 GGACATTGTAAATTTTGTAACTGAAAGGACAGCAAGTTATATCGAATTCC  
 CCTGTAACATTTAAACATTGACTTTCCTGTCGTTCAATATAGCTTAAGG  
 1851 TGCA  
 ACGTC

**FIGURE 6A**

## FIGURE 6B

WT preprorin linker

primer THERM-1

5'- AGGGAATTGCTTCTTTTATGCTGATGTTGTATG -3'

\*\*\*

TCTTTGCTTATAAGGCCAGTGCTGCCAAATTTTAAT  
AGAAACGAATATTCCGGTCACACCGTTTAAATTA

\*\*\*

GGTGGTAGCAGTGTCAAACTACACCTACTTTCCCTACAC -5'

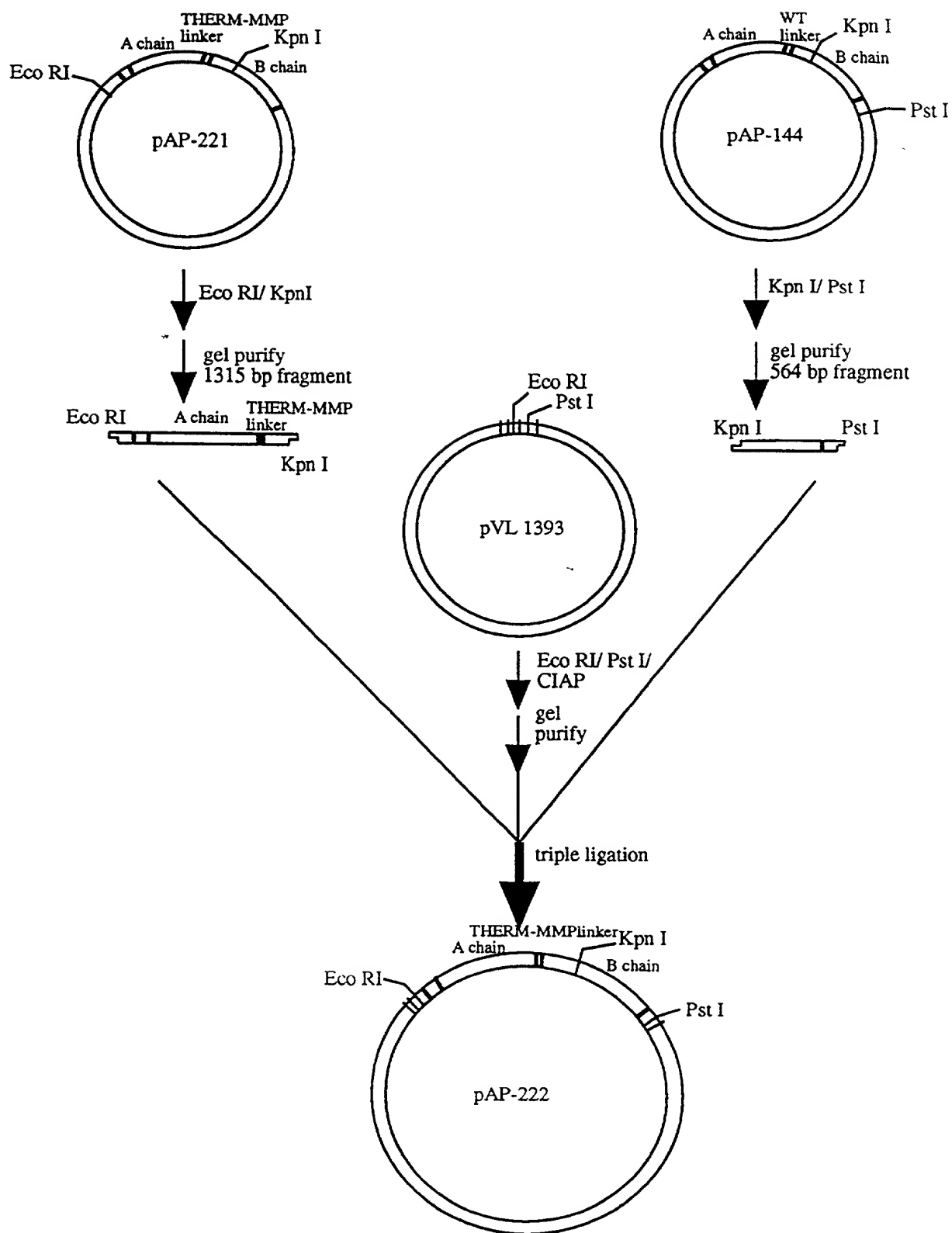
3'-

primer THERM-2

PCR mutagenesis  
ligate with pBluescript SK

pAP 221 linker  
(THERM-MMP variant)  
New Cleavage Site

GATGTGGATGAAAGGGATGTGAGGGAATTGCTTCTTTTA  
CTACACCTACTTTCCCTACACTCCCTTAAACGAAGAAAAAT

**FIGURE 6C**

**FIGURE 6D**

10 20 30 40 50  
 1 GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT  
 CTTAAGTACTTTGGCCCTCCTTTATGATAACATTATACCTACATACGTCA  
 51 GGCAACATGGCTTTGTTTTGGATCCACCTCAGGGTGGTCTTTCACATTAG  
 CCGTTGTACCGAAACAAAACCTAGGTGGAGTCCCACCAGAAAGTGTAAATC  
 101 AGGATAACAACATATTCCCCAAACAATACCCAATTATAAACTTTACCACA  
 TCCTATTGTTGTATAAGGGGTTTGTATGGGTAAATATTTGAAATGGTGT  
 151 GCGGGTGCCACTGTGCAAAGCTACACAACTTTATCAGAGCTGTTTCGCGG  
 CGCCACGGTGACACGTTTCGATGTGTTTGAAATAGTCTCGACAAGCGCC  
 201 TCGTTTAACAACCTGGAGCTGATGTGAGACATGATATACCAGTGTTCGCAA  
 ACGCAAATGTGTGACCTCGACTACACTCTGTACTATATGGTCACAACGGTT  
 251 ACAGAGTTGGTTTGCCTATAAACCACGGTTTATTTTAGTTGAACTCTCA  
 TGTCTCAACCAAACGGATATTTGGTTGCCAAATAAAATCAACTTGAGAGT  
 301 AATCATGCAGAGCTTCTGTACATTAGCGCTGGATGTCACCAATGCATA  
 TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT  
 351 TGTGGTCGGCTACCGTGCTGGAAATAGCGCATATTTCTTTCATCCTGACA  
 ACACCAGCCGATGGCACGACCTTTATCGCGTATAAAGAAAGTAGGACTGT  
 401 ATCAGGAAGATGCAGAAGCAATCACTCATCTTTTCACTGATGTTCAAAT  
 TAGTCCTTCTACGTCTTCGTTAGTGAGTAGAAAAGTGACTACAAGTTTTA  
 451 CGATATACATTTCGCCCTTTGGTGGTAATTATGATAGACTTGAACAACCTGC  
 GCTATATGTAAGCGGAAACCACCATTAATACTATCTGAACCTGTTGAACG  
 501 TGGTAATCTGAGAGAAAATATCGAGTTGGGAAATGGTCCACTAGAGGAGG  
 ACCATTAGACTCTCTTTTATAGCTCAACCCCTTACCAGGTGATCTCCTCC  
 551 CTATCTCAGCGCTTTATTATTACAGTACTGGTGGCACTCAGCTTCCAAC  
 GATAGAGTTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA  
 601 CTGGCTCGTTCCTTTATAATTTGCATCCAAATGATTTCAGAAGCAGCAAG  
 GACCGAGCAAGGAAATATTAAACGTAGGTTTACTAAAGTCTTCGTCGTTT  
 651 ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA  
 TAAGGTTATATAACTCCCTCTTTACGCGTGCTCTTAATCCATGTTGGCCT  
 701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGGAGA  
 CTAGACGTGGTCTAGGATCGCATTAATGTGAACCTTTATCAACCCCTCT  
 751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTTGCTAGTCCAAT  
 GAAAGGTGACGTTAAGTTCTCAGATTGGTTCTCGGAAACGATCAGGTTA  
 801 TCAACTGCAAAGACGTAATGGTTCCAAATTCAGTGTGTACGATGTGAGTA  
 AGTTGACGTTTCTGCATTACCAAGGTTTAAGTCACACATGCTACACTCAT  
 851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGCACCTCCACCA  
 ATAATTAGGGATAGTATCGAGAGTACCACATATCTACGCGTGGAGGTGGT  
 901 TCGTCACAGTTTGATGTGGATGAAAGGGATGTGAGGGAATTTGCTTCTTT  
 AGCAGTGTCAAACCTACACCTACTTCCCTACACTCCCTTAAACGAAGAAA  
 951 TTTAGCTGATGTTTGTATGGATCCTGAGCCCATAGTGCGTATCGTAGGTC

**FIGURE 6D (CONT'D)**

AAATCGACTACAAACATACCTAGGACTCGGGTATCACGCATAGCATCCAG

1001 GAAATGGTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAAC  
CTTTACCAGATACACAAC TACAATCCCTACCTTCTAAGGTGTTGCCTTTG

1051 GCAATACAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTG  
CGTTATGTCAACACCGGTACGTTTCAAGTTATGTCTACGTTTAGTCGAGAC

1101 GACTTTGAAAAGAGACAATACTATTCGATCTAATGGAAAGTGTTTAACTA  
CTGAAACTTTTCTCTGTTATGATAAGCTAGATTACCTTTCACAAATTGAT

1151 CTTACGGGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACT  
GAATGCCCATGTCAGGCCCTCAGATACACTACTAGATACTAACGTTATGA

1201 GCTGCAACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCAT  
CGACGTTGACTACGGTGGGCGACCGTTTATACCCTATTACCTTGGTAGTA

1251 AAATCCCAGATCTAGTCTAGTTTTAGCAGCGACATCAGGGAACAGTGGA  
TTTAGGGTCTAGATCAGATCAAAATCGTCGCTGTAGTCCCTTGTCAACCAT

1301 CCACACTTACAGTGCAAACCAACATTTATGCCGTTAGTCAAGGTTGGCTT  
GGTGTGAATGTCACGTTTGGTTGTAAATACGGCAATCAGTTCCAACCGAA

1351 CCTACTAATAATACACAACCTTTTGTGTTACAACCATTGTTGGGCTATATGG  
GGATGATTATTATGTGTTGGAAAACAATGTTGGTAAACAACCCGATATACC

1401 TCTGTGCTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCA  
AGACACGAACGTTTCGTTTATCACCTGTTTCATACCTATCTCCTGACATCGT

1451 GTGAAAAGGCTGAACAACAGTGGGCTCTTTATGCAGATGGTTCAATACGT  
CACTTTTCCGACTTGTGTCACCCGAGAAATACGTCTACCAAGTTATGCA

1501 CCTCAGCAAAACCGAGATAATTGCCTTACAAGTGATTCTAATATACGGGA  
GGAGTCGTTTGGCTCTATTAACGGAATGTTCACTAAGATTATATGCCCT

1551 AACAGTTGTTAAGATCCTCTCTTGTGGCCCTGCATCCTCTGGCCAACGAT  
TTGTCAACAATTCTAGGAGAGAACACCGGGACGTAGGAGACCGGTTGCTA

1601 GGATGTTCAAGAATGATGGAACCATTTTAAATTTGTATAGTGGATTGGTG  
CCTACAAGTTCTTACTACCTTGGTAAAATTTAAACATATCACCTAACCAC

1651 TTAGATGTGAGGCGATCGGATCCGAGCCTTAAACAAATCATTCTTTACCC  
AATCTACACTCCGCTAGCCTAGGCTCGGAATTTGTTTAGTAAGAAATGGG

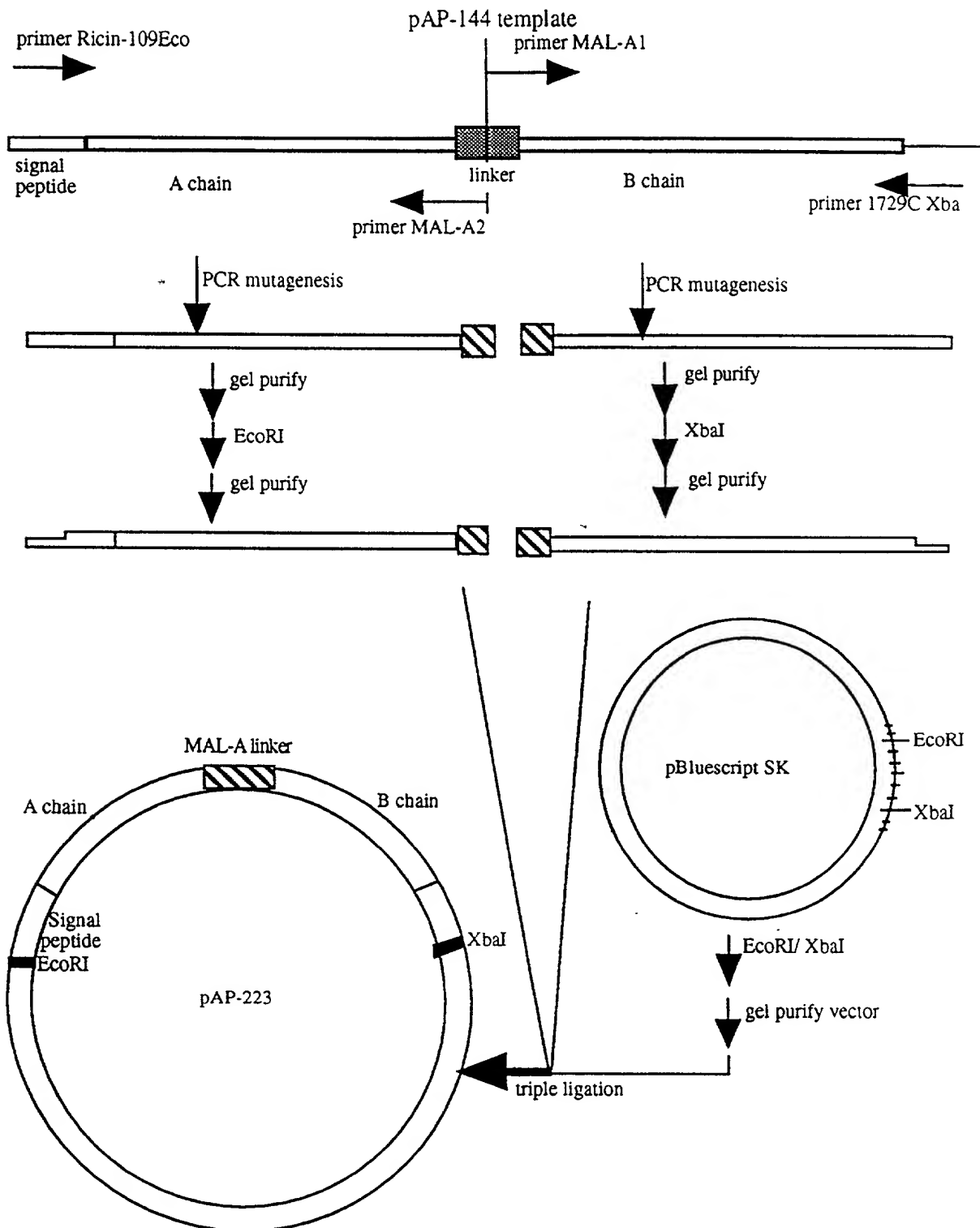
1701 TCTCCATGGTGACCCAAACCAATATGGTTACCATTATTTTGATAGACAG  
AGAGGTACCACTGGGTTTGGTTTATACCAATGGTAATAAACTATCTGTC

1751 ATTACTCTCTTGCAAGTGTGTGTCTGCCATGAAAATAGATGGCTTAA  
TAATGAGAGAACGTCACACACACAGGACGGTACTTTTATCTACCGAATTT

1801 TAAAAAGGACATTGTAAATTTTGTAAGTGAAGGACAGCAAGTTATATCG  
ATTTTTCTGTAAACATTTAAACATTGACTTTCCTGTCGTTCAATATAGC

1851 AATTCCTGCAG  
TTAAGGACGTC



**FIGURE 7A**

## FIGURE 7B

WT preprorin linker

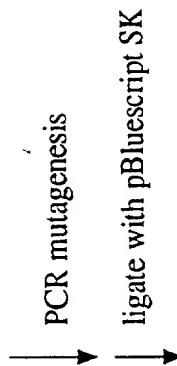
primer MAL-A1

5' - AATTATGATGAAGAGGATGCTGATGTTGTATG -3'  
\*\*\*\*\*

TCTTTGCTTATAAGGCCAGTGGTGCCAAATTTTAAT  
AGAAACGAATATTCCTGGTACACACGGTTTAAATA  
\*\*\*\*\*

3' - GGTAGCAGTGTCAAAGTCCCAAGTTAACGTC -5'

primer MAL-A2

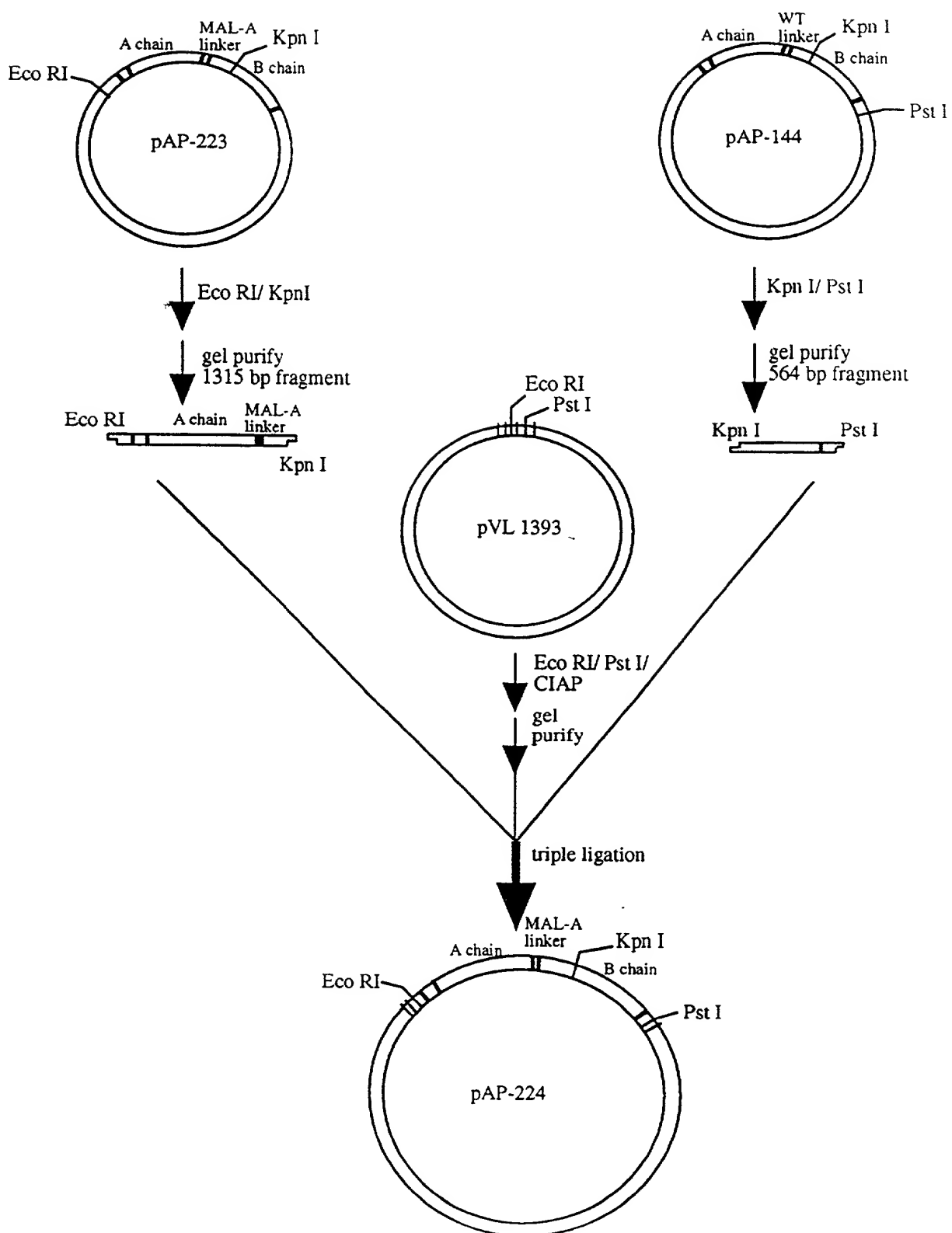


pAP 223 linker  
(MAL-A variant)

CAGGTGGTTCAATTCCAGAATTATGATGAAGAGGAT  
GTCCACCAAGTTAAGTCTTAATACTACTTCTCCTA

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**FIGURE 7C**



## FIGURE 7D

10 20 30 40 50

1 GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT  
CTTAAGTACTTTGGCCCTCCTTTATGATAACATTATACCTACATACGTCA

51 GGCAACATGGCTTTGTTTTGGATCCACCTCAGGGTGGTCTTTCACATTAG  
CCGTTGTACCGAAACAAAACCTAGGTGGAGTCCCACCAGAAAGTGAATC

101 AGGATAACAACATATTCCCCAAACAATACCCAATTATAAACTTTACCACA  
TCCTATTGTTGTATAAGGGGTTTGTTATGGGTTAATATTTGAAATGGTGT

151 GCGGGTGCCACTGTGCAAAGCTACACAACTTTATCAGAGCTGTTTCGCGG  
CGCCACGGTGACACGTTTCGATGTGTTTGAAATAGTCTCGACAAGCGCC

201 TCGTTTAACAACTGGAGCTGATGTGAGACATGATATACCAGTGTTCGCAA  
AGCAAATTGTTGACCTCGACTACACTCTGTACTATATGGTCACAACGGTT

251 ACAGAGTTGGTTTGCCTATAAACCAACGGTTTATTTTAGTTGAACTCTCA  
TGTCTCAACCAAACGGATATTTGGTTGCCAAATAAAATCAACTTGAGAGT

301 AATCATGCAGAGCTTCTGTACATTAGCGCTGGATGTCACCAATGCATA  
TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT

351 TGTGGTCGGCTACCGTGCTGGAAATAGCGCATATTTCTTTCATCCTGACA  
ACACCAGCCGATGGCACGACCTTTATCGCGTATAAAGAAAGTAGGACTGT

401 ATCAGGAAGATGCAGAAGCAATCACTCATCTTTTCACTGATGTTCAAAAT  
TAGTCCTTCTACGTCTTCGTTAGTGAGTAGAAAAGTGACTACAAGTTTAA

451 CGATATACATTTCGCCTTTGGTGGTAATTATGATAGACTTGAACAACCTTGC  
GCTATATGTAAGCGGAAACCACCATTAACTACTATCTGAACTTGTGTAACG

501 TGGTAATCTGAGAGAAAATATCGAGTTGGGAAATGGTCCACTAGAGGAGG  
ACCATTAGACTCTCTTTTATAGCTCAACCCTTTACCAGGTGATCTCCTCC

551 CTATCTCAGCGCTTTATTATTACAGTACTGGTGGCACTCAGCTTCCAAC  
GATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA

601 CTGGCTCGTTTCCTTTATAATTTGCATCCAAATGATTTGAGAAGCAGCAAG  
GACCGAGCAAGGAAATATTAACGTTAGGTTTACTAAAGTCTTCGTCGTTTC

651 ATTCCAATATATTGAGGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA  
TAAGGTTATATAACTCCCTCTTTACGCGTGCTCTTAATCCATGTTGGCCT

701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGGAGA  
CTAGACGTGGTCTAGGATCGCATTAATGTGAACTCTTATCAACCCCTCT

751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTTGCTAGTCCAAT  
GAAAGGTGACGTTAAGTTCAGATTGGTTTCCTCGGAAACGATCAGGTTA

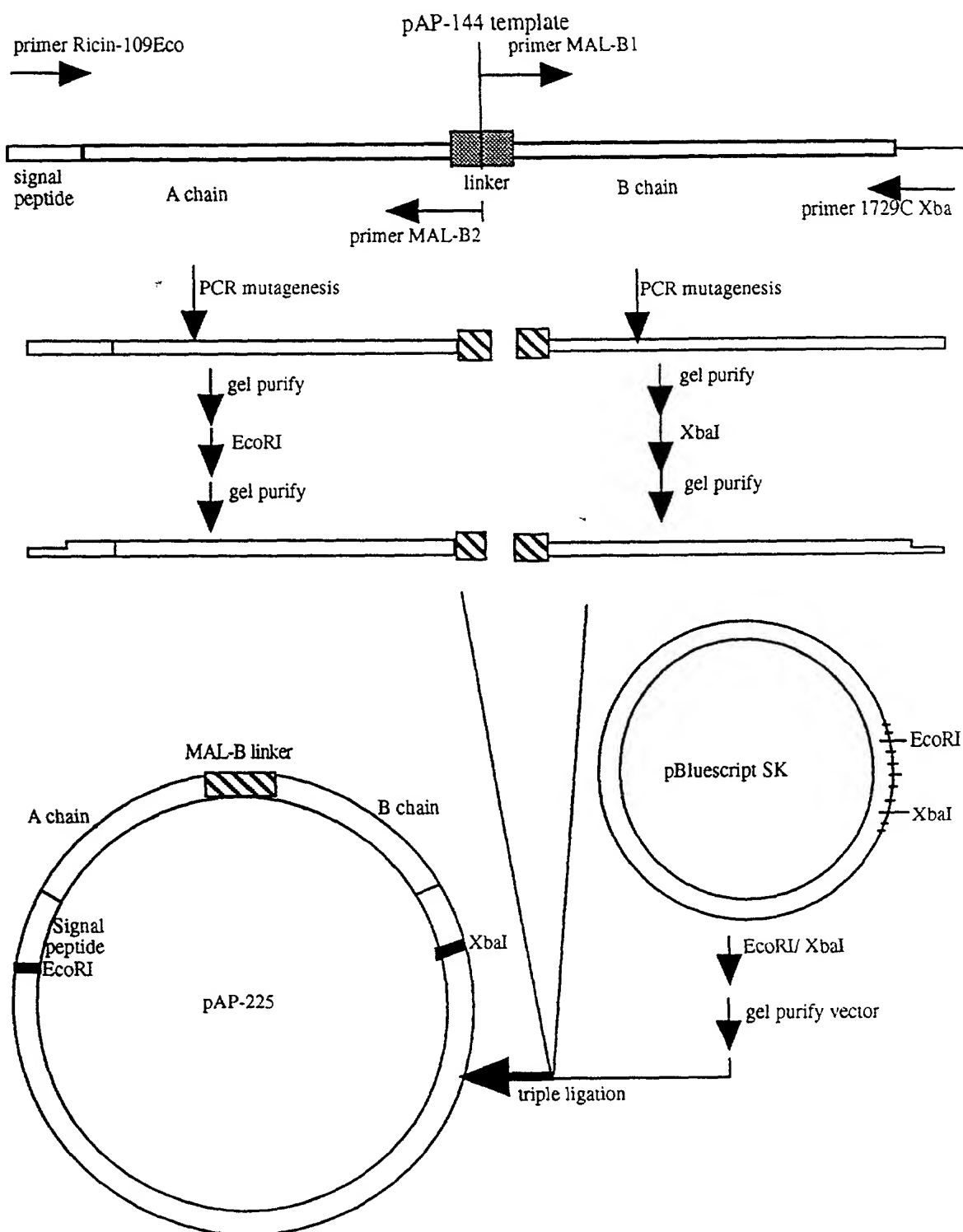
801 TCAACTGCAAAGACGTAATGGTTCCAAATTCAGTGTGTACGATGTGAGTA  
AGTTGACGTTTCTGCATTACCAAGGTTTAAAGTCACACATGCTACACTCAT

851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGCACCTCCACCA  
ATAATTAGGGATAGTATCGAGAGTACCACATATCTACGCGTGGAGGTGGT

901 TCGTCACAGTTTTCAGGTGGTTCAATTGCAGAATTATGATGAAGAGGATGC  
AGCAGTGTCAAAGTCCACCAAGTTAACGTCCTTAATACTACTTCTCCTACG

**FIGURE 7D (CONT'D)**

951 TGATGTTTGTATGGATCCTGAGCCCATAGTGCGTATCGTAGGTCGAAATG  
 ACTACAAACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTTAC  
 1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA  
 CAGATACACAACACTACAATCCCTACCTTCTAAGGTGTTGCCTTTGCGTTAT  
 1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAAATCAGCTCTGGACTTT  
 GTCAACACCCGTACGTTTCAGATTATGTCTACGTTTAGTCGAGACCTGAAA  
 1101 GAAAAGAGACAATACTATTTCGATCTAATGGAAAGTGTTTAACTACTTACG  
 CTTTTCTCTGTTATGATAAGCTAGATTACCTTTTACAAAATTGATGAATGC  
 1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA  
 CCATGTCAGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT  
 1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC  
 TGA CTACGTTGGGCGACCGTTTATACCCTATTACCTTGGTAGTATTTAGG  
 1251 CAGATCTAGTCTAGTTTTAGCAGCGACATCAGGGAACAGTGGTACCACAC  
 GTCTAGATCAGATCAAAATCGTCGCTGTAGTCCCTTGTCAACCATGGTGTG  
 1301 TTACAGTGCAAACCAACATTTATGCCGTTAGTCAAGGTTGGCTTCCTACT  
 AATGTCACGTTTGGTTGTAAATACGGCAATCAGTTCCAACCGAAGGATGA  
 1351 AATAATACACAACCTTTTGTTCACAACCATTGTTGGGCTATATGGTCTGTG  
 TTATTATGTGTTGGAAAACAATGTTGGTAACAACCCGATATACCAGACAC  
 1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA  
 GAACGTTTCGTTTATCACCTGTTTCATACCTATCTCCTGACATCGTCACTTT  
 1451 AGGCTGAACAACAGTGGGCTCTTTATGCAGATGGTTCAATACGTCCTCAG  
 TCCGACTTGTTGTCACCCGAGAAATACGCTTACCAAGTTATGCAGGAGTC  
 1501 CAAAACCGAGATAATTGCCTTACAAGTGATTCTAATATACGGGAAACAGT  
 GTTTTGGCTCTATTAAACGGAATGTTCACTAAGATTATATGCCCTTTGTCA  
 1551 TGTTAAGATCCTCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT  
 ACAATTCTAGGAGAGAACACCGGGACGTAGGAGACCGGTTGCTACCTACA  
 1601 TCAAGAATGATGGAACCATTTTAAATTTGTATAGTGGATTGGTGTTAGAT  
 AGTTCCTTACTACCTTGGTAAATTTAAACATATCACCTAACCAACAATCTA  
 1651 GTGAGGCGATCGGATCCGAGCCTTAAACAAATCATCTTTACCCTCTCCA  
 CACTCCGCTAGCCTAGGCTCGGAATTTGTTTAGTAAGAAATGGGAGAGGT  
 1701 TGGTGACCCAAACCAAATATGGTTACCATTATTTTGATAGACAGATTACT  
 ACCACTGGGTTTGGTTTATACCAATGGTAATAAACTATCTGTCTAATGA  
 1751 CTCTTGCAGTGTGTGTGTCTCTGCCATGAAAATAGATGGCTTAAATAAAAA  
 GAGAACGTCACACACACAGGACGGTACTTTTATCTACCGAATTTATTTTT  
 1801 GGACATTGTAAATTTTGTAACTGAAAGGACAGCAAGTTATATCGAATTCC  
 CCTGTAAACATTTAAACATTGACTTTCCTGTCGTTCAATATAGCTTAAGG  
 1851 TGCAG  
 ACGTC

**FIGURE 8A**

# FIGURE 8B

WT preprorin linker

primer MAL-B1

5' - TCGGAGGACAATGATGAAGCTGATGTTGTATG -3'

TCTTTGCTTATAAGGCCAGTGGTGCCAAATTTTAAT  
AGAAACGAAATATTCCGGTCCACCACGGTTTAAAAATTA

3' - GGTAGCAGTGTCAAAAACGGCTAAAAGCCCCCTT -5'

primer MAL-B2

PCR mutagenesis

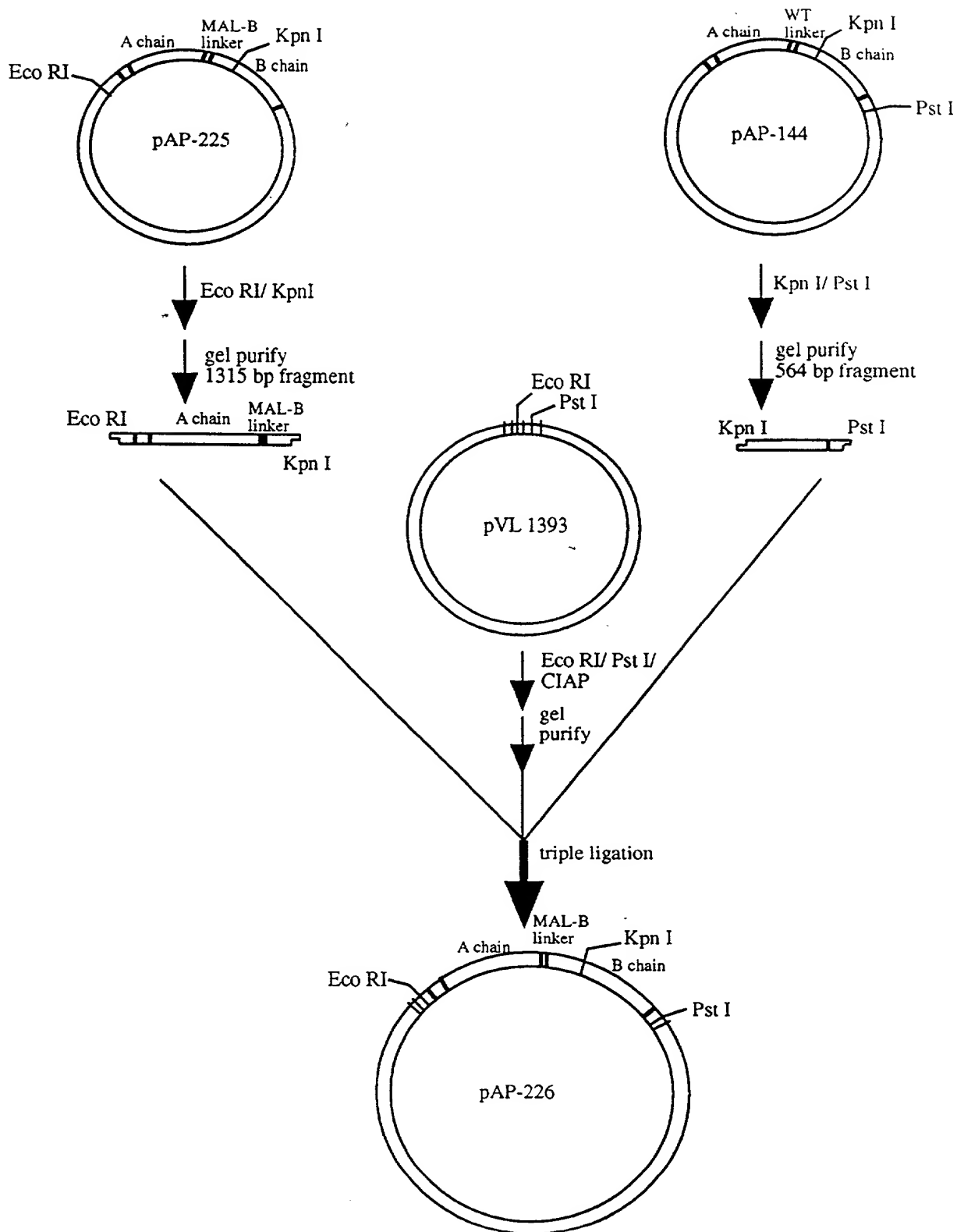
ligate with pBluescript SK

pAP 225 linker  
(MAL-B variant)

TTGCCGATTTTCGGGGAATCGGAGGACAATGATGAA  
AACGGCTAAAAGCCCCCTTAGCCTCCTGTACTACTT

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**FIGURE 8C**



00440" T5T560



**FIGURE 8D**

10 20 30 40 50  
 1 GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT  
 CTTAAGTACTTTGGCCCTCCTTTATGATAACATTATACCTACATACGTCA  
 51 GGCAACATGGCTTTGTTTTGGATCCACCTCAGGGTGGTCTTTACATTAG  
 CCGTTGTACCGAAACAAAACCTAGGTGGAGTCCCACCAGAAAGTGTAAATC  
 101 AGGATAACAACATATTCCCCAAACAATACCCAATTATAAACTTTACCACA  
 TCCTATTGTTGTATAAGGGGTTGTTATGGGTAAATATTTGAAATGGTGT  
 151 GCGGGTGCCACTGTGCAAAGCTACACAACTTTATCAGAGCTGTTTCGCGG  
 CGCCACGGTGACACGTTTCGATGTGTTTGAAATAGTCTCGACAAGCGCC  
 201 TCGTTTAAACAACTGGAGCTGATGTGAGACATGATATACCAGTGTGCCAA  
 AGCAAATTGTTGACCTCGACTACACTCTGTACTATATGGTCACAACGGTT  
 251 ACAGAGTTGGTTTGCCTATAAACCAACGGTTTATTTTAGTTGAACTCTCA  
 TGTCTCAACCAAACGGATATTTGGTTGCCAAATAAAATCAACTTGAGAGT  
 301 AATCATGCAGAGCTTCTGTTACATTAGCGCTGGATGTCACCAATGCATA  
 TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT  
 351 TGTGGTCGGCTACCGTGTGGAATAGCGCATATTTCTTTCATCCTGACA  
 ACACCAGCCGATGGCACGACCTTTATCGCGTATAAAGAAAGTAGGACTGT  
 401 ATCAGGAAGATGCAGAAGCAATCACTCATCTTTTCACTGATGTTCAAAT  
 TAGTCCCTCTACGTCTTCGTTAGTGAGTAGAAAAGTGACTACAAGTTTTA  
 451 CGATATACATTTCGCCCTTTGGTGGTAATTATGATAGACTTGAACAACTTGC  
 GCTATATGTAAGCGGAAACCACCATTAACTATCTGAAC TTGTTGAACG  
 501 TGGTAATCTGAGAGAAAATATCGAGTTGGGAAATGGTCCACTAGAGGAGG  
 ACCATTAGACTCTCTTTTATAGCTCAACCCTTTACCAGGTGATCTCCTCC  
 551 CTATCTCAGCGCTTTATTATTACAGTACTGGTGGCACTCAGCTTCCAAC  
 GATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA  
 601 CTGGCTCGTTCCCTTTATAATTTGCATCCAAATGATTTCAGAAGCAGCAAG  
 GACCGAGCAAGGAAATATTAAACGTAGGTTTACTAAAGTCTTCGTCTGTT  
 651 ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA  
 TAAGGTTATATAACTCCCTCTTTACGCGTGCTCTTAATCCATGTTGGCCT  
 701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGGAGA  
 CTAGACGTGGTCTAGGATCGCATTAATGTGAACCTTATCAACCCCTCT  
 751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTTGCTAGTCCAAT  
 GAAAGGTGACGTTAAGTTCTCAGATTGGTTTCCTCGGAAACGATCAGGTTA  
 801 TCAACTGCAAAGACGTAATGGTTCCAAATTCAGTGTGTACGATGTGAGTA  
 AGTTGACGTTTCTGCATTACCAAGGTTTAAAGTCACACATGCTACACTCAT  
 851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGCACCTCCACCA  
 ATAATTAGGGATAGTATCGAGAGTACCACATATCTACGCGTGGAGGTGGT  
 901 TCGTCACAGTTTTTGCCGATTTTCGGGGAATCGGAGGACAATGATGAAGC  
 AGCAGTGTCAAAAACGGCTAAAAGCCCTTAGCCTCCTGTTACTACTTCG

**FIGURE 8D (CONT'D)**

951 TGATGTTTGTATGGATCCTGAGCCCATAGTGC GTATCGTAGGTCGAAATG  
ACTACAAACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTTAC

1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA  
CAGATACACAACCTACAATCCCTACCTTCTAAGGTGTTGCCTTTGCGTTAT

1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT  
GTCAACACCGGTACGTTTACAGATTATGTCTACGTTTAGTCGAGACCTGAAA

1101 GAAAAGAGACAATACTATTTCGATCTAATGGAAAGTGTTTAACTACTTACG  
CTTTTCTCTGTTATGATAAGCTAGATTACCTTTCACAAATTGATGAATGC

1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA  
CCATGTACGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT

1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC  
TGACTACGGTGGGCGACCGTTTATACCCTATTACCTTGGTAGTATTTAGG

1251 CAGATCTAGTCTAGTTTTAGCAGCGACATCAGGGAACAGTGGTACCACAC  
GTCTAGATCAGATCAAAATCGTCGCTGTAGTCCCTTGTCAACCATGGTGTG

1301 TTACAGTGCAAACCAACATTTATGCCGTTAGTCAAGGTTGGCTTCCTACT  
AATGTACGTTTGGTTGTAAATACGGCAATCAGTTCCAACCGAAGGATGA

1351 AATAATACACAACCTTTTGTACAAACCATTGTTGGGCTATATGGTCTGTG  
TTATTATGTGTTGGAAAACAATGTTGGTAACAACCCGATATACCAGACAC

1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA  
GAACGTTTCGTTTATCACCTGTTTACATCCTATCTCCTGACATCGTCACTTT

1451 AGGCTGAACAACAGTGGGCTCTTTATGCAGATGGTTCAATACGTCCTCAG  
TCCGACTTGTGTGTCACCCGAGAAATACGTCTACCAAGTTATGCAGGAGTC

1501 CAAAACCGAGATAATTGCCTTACAAGTGATTCTAATATACGGGAAACAGT  
GTTTTGGCTCTATTAACGGAATGTTCACTAAGATTATATGCCCTTTGTCA

1551 TGTTAAGATCCTCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT  
ACAATTCTAGGAGAGAAACACCGGGACGTAGGAGACCGGTTGCTACCTACA

1601 TCAAGAATGATGGAACCATTTTAAATTTGTATAGTGGATTGGTGTAGAT  
AGTTCTTACTACCTTGGTAAAATTTAAACATATCACCTAACCAACAATCTA

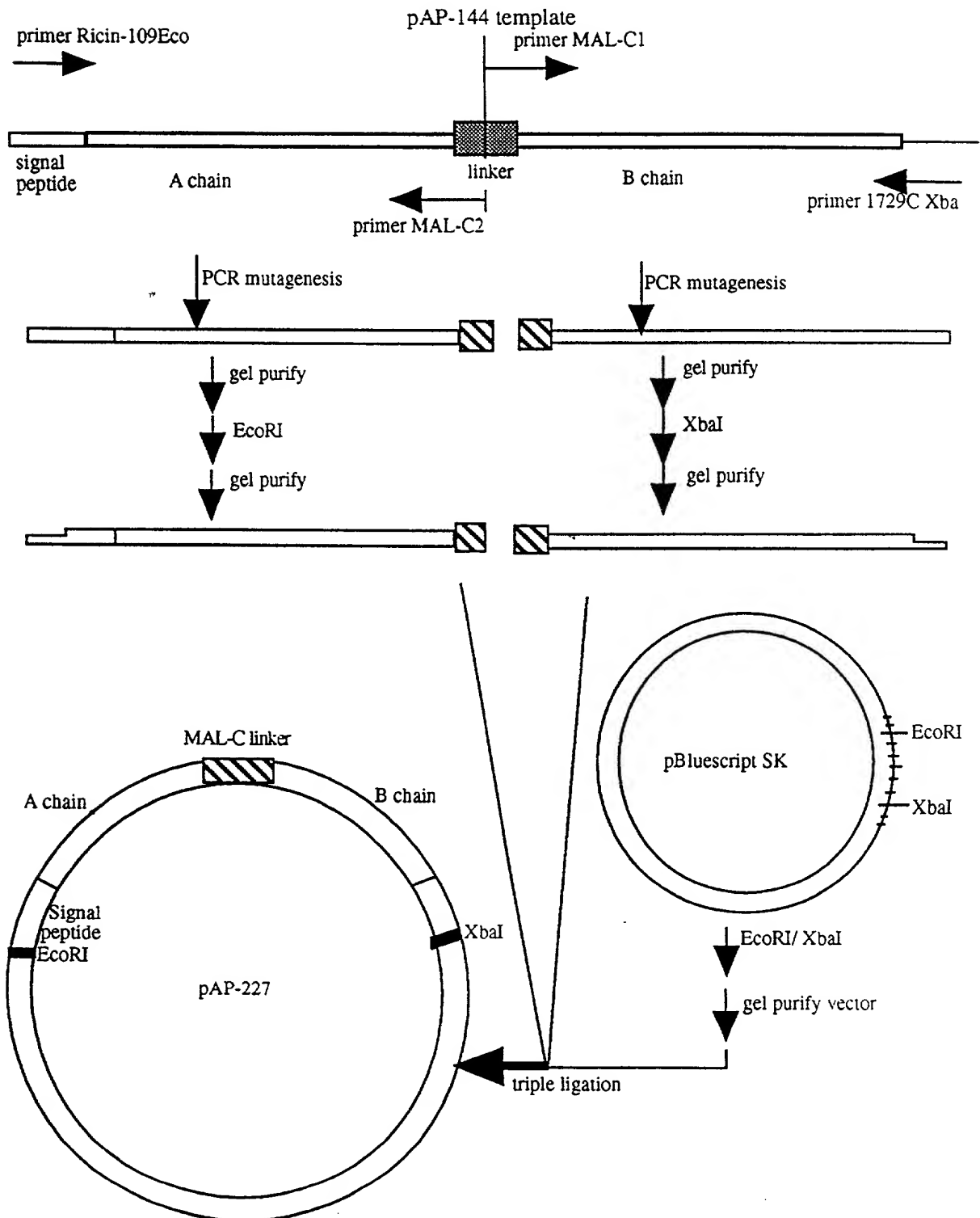
1651 GTGAGGCGATCGGATCCGAGCCTTAAACAAATCATTCCTTACCCTCTCCA  
CACTCCGCTAGCCTAGGCTCGGAATTTGTTTAGTAAGAAATGGGAGAGGT

1701 TGGTGACCCAAACCAATATGGTTACCATTATTTTGTATAGACAGATTACT  
ACCACTGGGTTTGGTTTATACCAATGGTAATAAACTATCTGTCTAATGA

1751 CTCTTGCAGTGTGTGTGTCCTGCCATGAAAAATAGATGGCTTAAATAAAAA  
GAGAACGTCACACACACAGGACGGTACTTTTATCTACCGAATTTATTTTT

1801 GGACATTGTAAATTTTGTAACTGAAAGGACAGCAAGTTATATCGAATTCC  
CCTGTAACATTTAAACATTGACTTTCCTGTCGTTCAATATAGCTTAAGG

1851 TGCAG  
ACGTC

**FIGURE 9A**

## FIGURE 9B

WT preprorin linker

primer MAL-C1

5' - GCGATATCAGTTACTATGGCTGATGTTGTATG -3'

TCTTTGCTTATAAGGCCAGTGGTGCCAAATTTTAAT  
AGAAACGAATATTCCTGGTACCCACGGTTTAAATTA

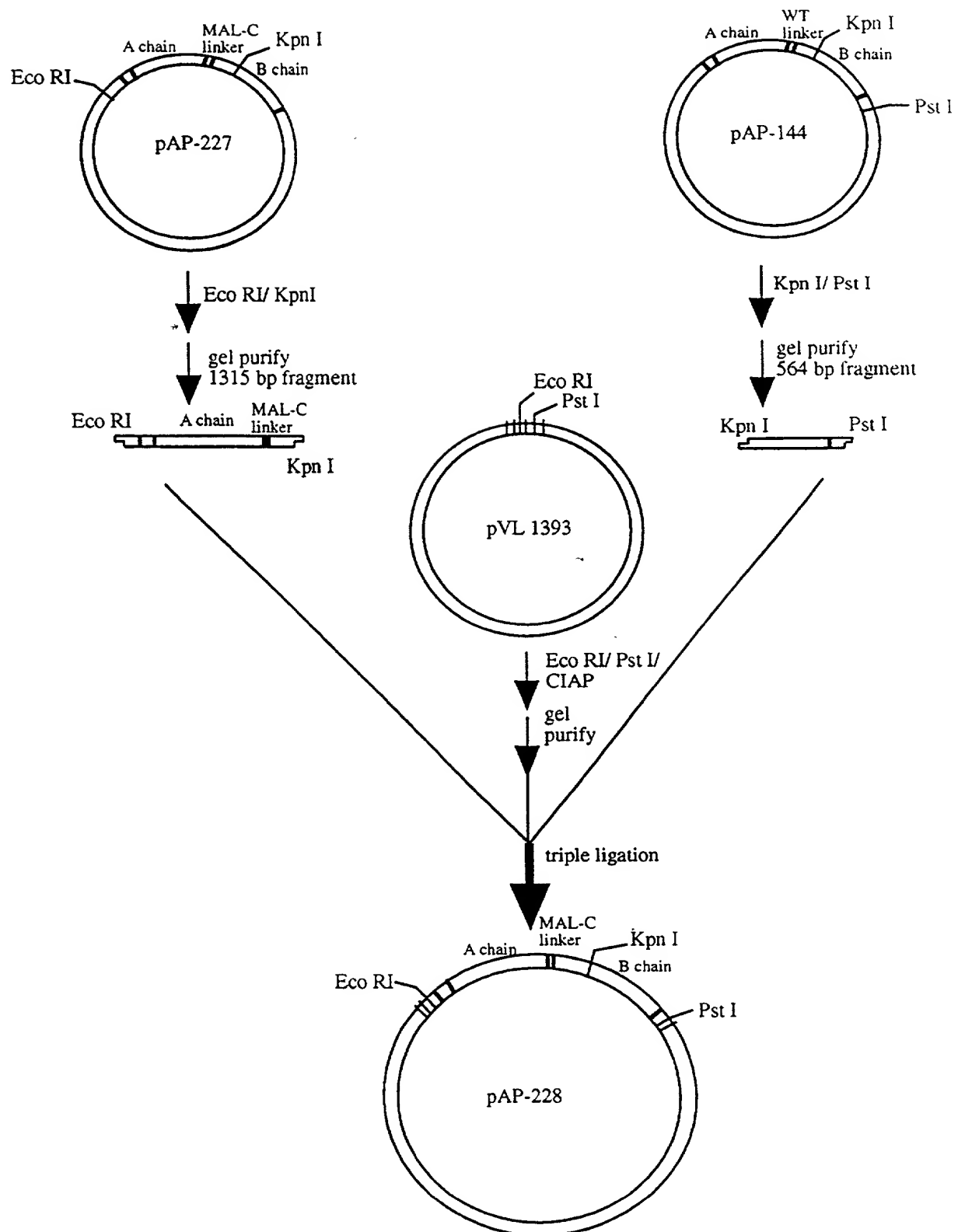
3' - GGTAGCAGTGTCAAAGTCCACCAATGTCCCCCTT -5'

primer MAL-C2

PCR mutagenesis  
ligate with pBluescript SK

pAP 227 linker  
(MAL-C variant)

CAGGTGTTACAGGGGAAGCGATATCAGTTACTATG  
GTCCACCAATGTCCCCCTTCGCTATAGTCAATGATAC

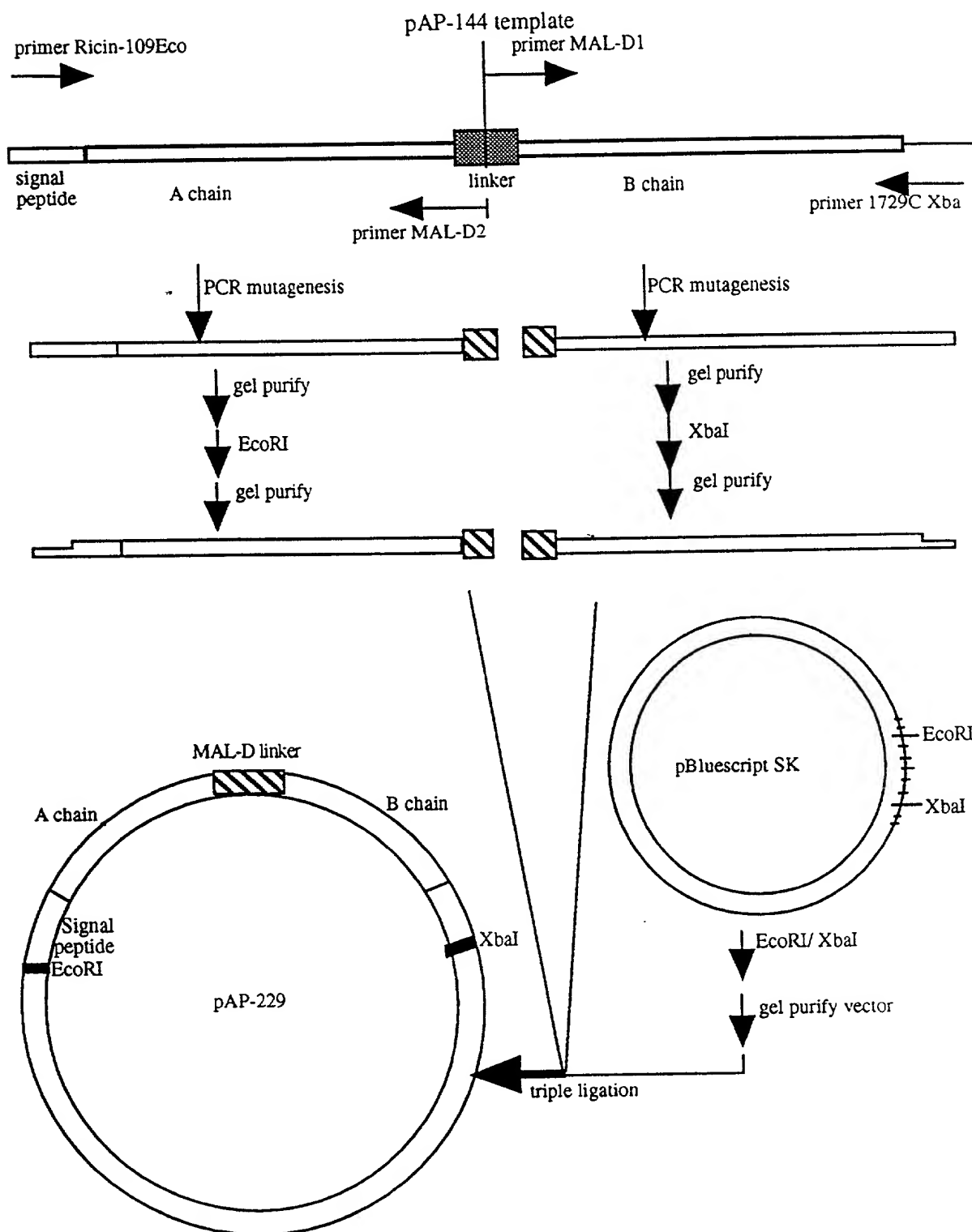
**FIGURE 9C**

**FIGURE 9D**

10 20 30 40 50  
 1 GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT  
 CTTAAGTACTTTGGCCCTCCTTTATGATAACATTATACCTACATACGTCA  
 51 GGCAACATGGCTTTGTTTTGGATCCACCTCAGGGTGGTCTTTTCACATTAG  
 CCGTTGTACCGAAACAAAACCTAGGTGGAGTCCCACCAGAAAGTGTAAATC  
 101 AGGATAACAACATATTCCCCAAACAATACCCAATTATAAACTTTACCACA  
 TCCTATTGTTGTATAAGGGGTTTGTATGGGTTAATATTTGAAATGGTGT  
 151 GCGGGTGCCACTGTGCAAAGCTACACAACTTTATCAGAGCTGTTTCGCGG  
 CGCCACGGTGACACGTTTCGATGTGTTTGAAATAGTCTCGACAAGCGCC  
 201 TCGTTTAACAACCTGGAGCTGATGTGAGACATGATATACAGTGTGCCAA  
 AGCAAATTGTTGACCTCGACTACACTCTGTACTATATGGTCACAACGGTT  
 251 ACAGAGTTGGTTTGCCTATAAACCAACGGTTTATTTTAGTTGAACTCTCA  
 TGTCTCAACCAAACGGATATTTGGTTGCCAAATAAAATCAACTTGAGAGT  
 301 AATCATGCAGAGCTTTCTGTTACATTAGCGCTGGATGTCACCAATGCATA  
 TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT  
 351 TGTGGTCGGCTACCGTGCTGGAAATAGCGCATATTTCTTTTCATCCTGACA  
 ACACCAGCCGATGGGCACGACCTTTATCGCGTATAAAGAAAGTAGGACTGT  
 401 ATCAGGAAGATGCAGAAGCAATCACTCATCTTTTCACTGATGTTCAAAAT  
 TAGTCTTCTACGTCTTCGTTAGTGAGTAGAAAAGTGACTACAAGTTTTA  
 451 CGATATACATTTCGCCTTTGGTGGTAATTATGATAGACTTGAACAACTTGC  
 GCTATATGTAAGCGGAAACCACCATTAACTATCTGAACTTGTGTAACG  
 501 TGGTAATCTGAGAGAAAATATCGAGTTGGGAAATGGTCCACTAGAGGAGG  
 ACCATTAGACTCTCTTTTATAGCTCAACCCTTTACCAGGTGATCTCCTCC  
 551 CTATCTCAGCGCTTTATTATTACAGTACTGGTGGCACTCAGCTTCCAAC  
 GATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA  
 601 CTGGCTCGTTCCTTTATAATTTGCATCCAAATGATTTTCAGAAGCAGCAAG  
 GACCGAGCAAGGAAATATTAAACGTAGGTTTACTAAAGTCTTCGTCGTTT  
 651 ATTCCAATATATTGAGGGAGAAAATGCGCACGAGAAATTAGGTACAACCGGA  
 TAAGGTTATATAACTCCCTCTTTACGCGTGCTCTTAATCCATGTTGGCCT  
 701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGGAGA  
 CTAGACGTGGTCTAGGATCGCATTAATGTGAACTCTTATCAACCCCTCT  
 751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTTGCTAGTCCAAT  
 GAAAGGTGACGTTAAGTTCTCAGATTGGTTCTCGGAAACGATCAGGTTA  
 801 TCAACTGCAAAGACGTAATGGTTCCAAATTCAGTGTGTACGATGTGAGTA  
 AGTTGACGTTTCTGCATTACCAAGGTTTAAAGTCACACATGCTACACTCAT  
 851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGCACCTCCACCA  
 ATAATTAGGGATAGTATCGAGAGTACCACATATCTACGCGTGGAGGTGGT  
 901 TCGTCACAGTTTCAGGTGGTTACAGGGGAAGCGATATCAGTTACTATGGC  
 AGCAGTGTCAAAGTCCACCAATGTCCCCTTCGCTATAGTCAATGATACCG

**FIGURE 9D (CONT'D)**

951 TGATGTTTGTATGGATCCTGAGCCCATAGTGCCTATCGTAGGTCGAAATG  
 ACTACAAACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTTAC  
 1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA  
 CAGATACACAACCTACAATCCCTACCTTCTAAGGTGTTGCCTTTGCGTTAT  
 1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT  
 GTCAACACCGGTACGTTTACAGATTATGTCTACGTTTAGTCGAGACCTGAAA  
 1101 GAAAAGAGACAATACTATTTCGATCTAATGGAAAGTGTTTAACTACTTACG  
 CTTTCTCTGTTATGATAAGCTAGATTACCTTTCACAAATTGATGAATGC  
 1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA  
 CCATGTCAGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT  
 1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC  
 TGACTACGGTGGGCGACCGTTTATACCTATTACCTTGGTAGTATTTAGG  
 1251 CAGATCTAGTCTAGTTTTAGCAGCGACATCAGGGAACAGTGGTACCACAC  
 GTCTAGATCAGATCAAAATCGTCGCTGTAGTCCCTTGTCAACCATGGTGTG  
 1301 TTACAGTGCAAACCAACATTTATGCCGTTAGTCAAGGTTGGCTTCCTACT  
 AATGTCACGTTTGGTTGTAAATACGGCAATCAGTTCCAACCGAAGGATGA  
 1351 AATAATACACAACCTTTTGTACAAACCATTTGTTGGGCTATATGGTCTGTG  
 TTATTATGTGTTGGAAAACAATGTTGGTAACAACCCGATATACCAGACAC  
 1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA  
 GAACGTTTCGTTTATCACCTGTTTCATACCTATCTCCTGACATCGTCACTTT  
 1451 AGGCTGAACAACAGTGGGCTCTTTATGCAGATGGTTCAATACGTCCTCAG  
 TCCGACTTGTGTGTCACCCGAGAAATACGTCTACCAAGTTATGCAGGAGTC  
 1501 CAAAACCGAGATAATTGCCTTACAAGTGATTCTAATATACGGGAAACAGT  
 GTTTTGGCTCTATTAACGGAATGTTCACTAAGATTATATGCCCTTTGTCA  
 1551 TGTTAAGATCCTCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT  
 ACAATTCTAGGAGAGAACACCGGGACGTAGGAGACCGGTTGCTACCTACA  
 1601 TCAAGAATGATGGAACCATTTTAAATTTGTATAGTGGATTGGTGTAGAT  
 AGTTCTTACTACCTTGGTAAAATTTAAACATATCACCTAACCAATCTA  
 1651 GTGAGGCGATCGGATCCGAGCCTTAAACAAATCATTCTTTACCCTCTCCA  
 CACTCCGCTAGCCTAGGCTCGGAATTTGTTTAGTAAGAAATGGGAGAGGT  
 1701 TGGTGACCCAAACCAAATATGGTTACCATTATTTTGATAGACAGATTACT  
 ACCACTGGGTTTGGTTTATACCAATGGTAATAAACTATCTGTCTAATGA  
 1751 CTCTTGCAGTGTGTGTGCTGCGCATGAAAATAGATGGCTTAAATAAAAA  
 GAGAACGTCACACACACAGGACGGTACTTTTATCTACCGAATTTATTTTT  
 1801 GGACATTGTAAATTTTGTAACTGAAAGGACAGCAAGTTATATCGAATTCC  
 CCTGTAACATTTAAACATTGACTTTCCTGTGCGTTCAATATAGCTTAAGG  
 1851 TGCAG  
 ACGTC

**FIGURE 10A**



## FIGURE 10B

WT preprorin linker

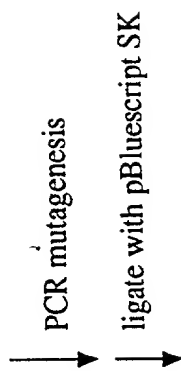
primer MAL-D1

5' - CTGTCGTTCCCTACTAATGCTGATGTTTGT -3'

TCTTTGCTTATAAGGCCAGTGGTGCCCAATTTTAAT  
AGAAACGAATATTCGGTACCCACGGTTTAAATAA

3' - GGTAGCAGTGTCAAACGAAACCTCTCTTGCAAG -5'

primer MAL-D2

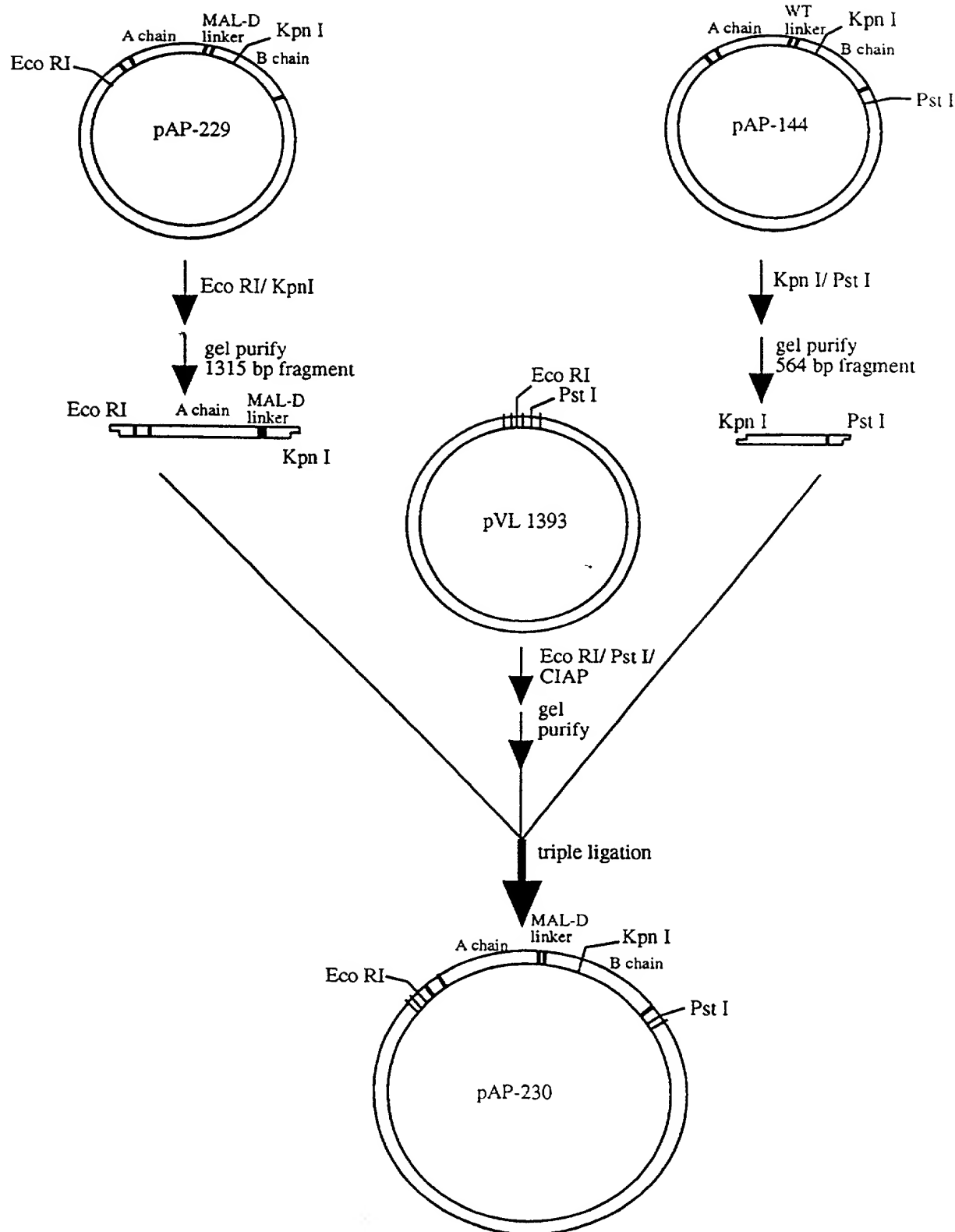


pAP 229 linker  
(MAL-D variant)

GCTTTGGAGAGAACGTTCCCTGTCGTTCCCTACTAAT  
CGAAACCTCTCTTGCAAGGACAGCAAGGATGATTA

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**FIGURE 10C**



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## FIGURE 10D

10 20 30 40 50

1 GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT  
CTTAAGTACTTTGGCCCTCCTTTATGATAACATTATACCTACATACGTCA

51 GGCAACATGGCTTTGTTTTGGATCCACCTCAGGGTGGTCTTTCACATTAG  
CCGTTGTACCGAAACAAACCTAGGTGGAGTCCCACCAGAAAGTGTAAATC

101 AGGATAACAACATATTCCCCAAACAATACCCAATTATAAACTTTACCACA  
TCCTATTGTTGTATAAGGGGTTTGTATGGGTAAATATTTGAAATGGTGT

151 GCGGGTGCCACTGTGCAAAGCTACACAACTTTATCAGAGCTGTTGCGGG  
CGCCCACGGTGACACGTTTCGATGTGTTTGAAATAGTCTCGACAAGCGCC

201 TCGTTTAAACAACTGGAGCTGATGTGAGACATGATATACCAGTGTGCCAA  
AGCAAATTGTTGACCTCGACTACACTCTGTACTATATGGTCACAACGGTT

251 ACAGAGTTGGTTTGCCTATAAACCAACGGTTTATTTTAGTTGAACTCTCA  
TGTCTCAACCAACGGATATTTGGTTGCCAAATAAAATCAACTTGAGAGT

301 AATCATGCAGAGCTTCTGTACATTAGCGCTGGATGTCACCAATGCATA  
TtagTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT

351 TGTGGTCGGCTACCGTGCTGGAAATAGCGCATATTTCTTTCATCCTGACA  
ACACCAGCCGATGGCAGACCTTTATCGCGTATAAAGAAAGTAGGACTGT

401 ATCAGGAAGATGCAGAAGCAATCACTCATCTTTTCACTGATGTTCAAAAT  
TAGTCCTTCTACGTCTTCGTTAGTGAGTAGAAAAGTGACTACAAGTTTTA

451 CGATATACATTTCGCCCTTTGGTGGTAATTATGATAGACTTGAACAACTGCG  
GCTATATGTAAGCGGAAACCACCATTAACTACTATCTGAACCTGTTGAACG

501 TGGTAATCTGAGAGAAAATATCGAGTTGGGAAATGGTCCACTAGAGGAGG  
ACCATTAGACTCTCTTTTATAGCTCAACCCTTTACCAGGTGATCTCCTCC

551 CTATCTCAGCGCTTTATTATTACAGTACTGGTGGCACTCAGCTTCCAAC  
GATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTGA

601 CTGGCTCGTTTCCTTTATAATTTGCATCCAAATGATTTTCAAGCAGCAAG  
GACCGAGCAAGGAAATATTAAACGTAGGTTTACTAAAGTCTTCGTCGTTC

651 ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA  
TAAGGTTATATAACTCCCTCTTTACGCGTGCTCTTAATCCATGTTGGCCT

701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGGAGA  
CTAGACGTGGTCTAGGATCGCATTAATGTGAACTCTTATCAACCCCTCT

751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTTGCTAGTCCAAT  
GAAAGGTGACGTTAAGTTCTCAGATTGGTTCCCTCGGAAACGATCAGGTGA

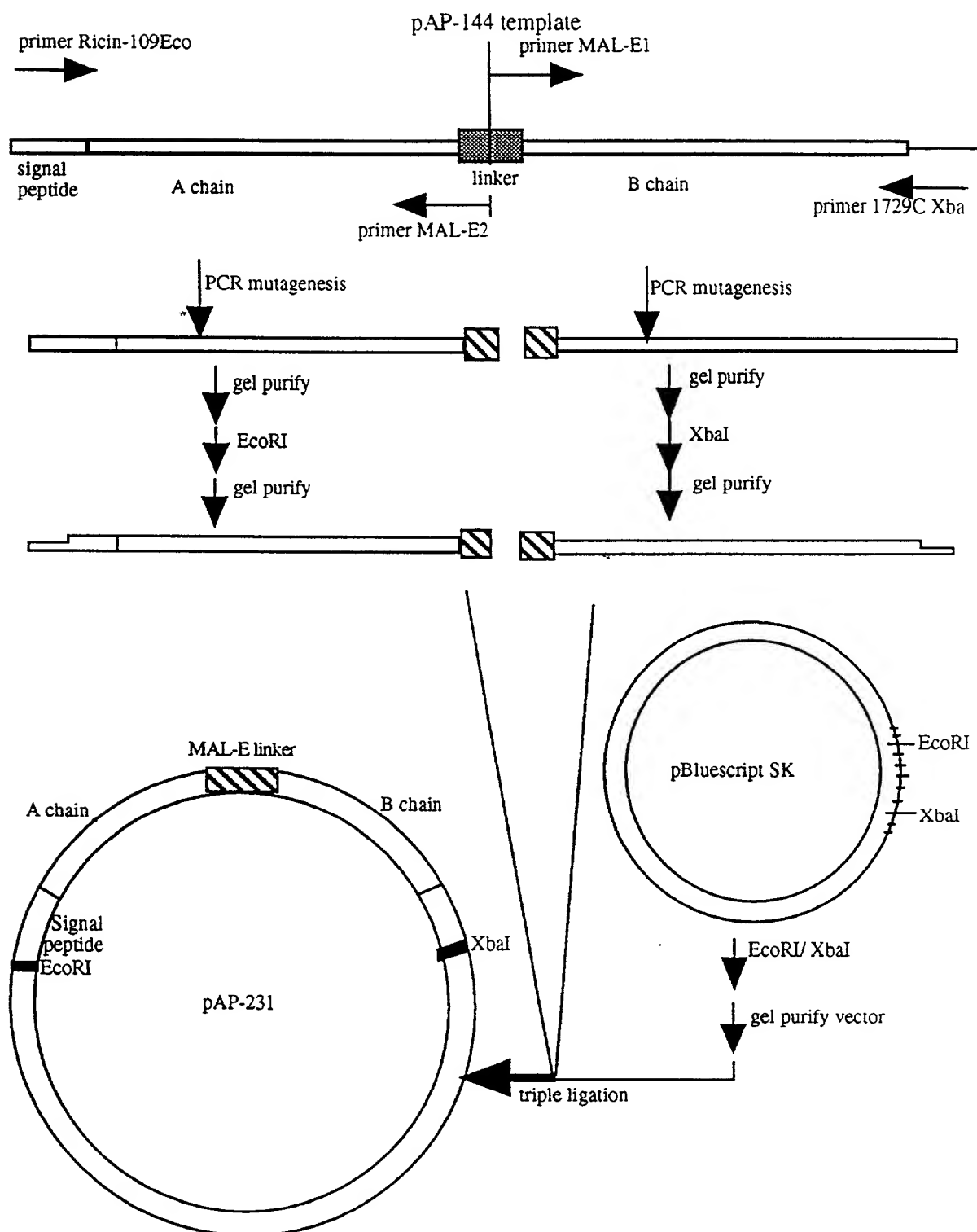
801 TCAACTGCAAAGACGTAATGGTTCCAAATTCAGTGTGTACGATGTGAGTA  
AGTTGACGTTTCTGCATTACCAAGGTTAAGTCACACATGCTACACTCAT

851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGCACCTCCACCA  
ATAATTAGGGATAGTATCGAGAGTACCACATATCTACGCGTGGAGGTGGT

901 TCGTCACAGTTTGCTTTGGAGAGAACGTTCTGTGCTTCCCTACTAATGC  
AGCAGTGTCAAACGAAACCTCTCTTGCAAGGACAGCAAGGGATGATTACG

**FIGURE 10D (CONT'D)**

951 TGATGTTTGTATGGATCCTGAGCCCATAGTGCGTATCGTAGGTCGAAATG  
 ACTACAAACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTTAC  
 1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA  
 CAGATACACAACCTACAATCCCTACCTTCTAAGGTGTTGCCTTTGCGTTAT  
 1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT  
 GTCAACACCGGTACGTTTACGATTATGTCTACGTTTAGTCGAGACCTGAAA  
 1101 GAAAAGAGACAATACTATTTCGATCTAATGGAAAAGTGTTTAACTACTTACG  
 CTTTTCTCTGTTATGATAAGCTAGATTACCTTTCACAAATTGATGAATGC  
 1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA  
 CCATGTACAGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT  
 1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC  
 TGACTACGGTGGGCGACCGTTTATACCCTATTACCTTGGTAGTATTTAGG  
 1251 CAGATCTAGTCTAGTTTTAGCAGCGACATCAGGGAACAGTGGTACCACAC  
 GTCTAGATCAGATCAAAATCGTCGCTGTAGTCCCTTGTCAACCATGGTGTG  
 1301 TTACAGTGCAAACCAACATTTATGCCGTTAGTCAAGGTTGGCTTCCTACT  
 AATGTCACGTTTGGTTGTAAATACGGCAATCAGTTCCAACCGAAGGATGA  
 1351 AATAATACACAACCTTTTGTACCAACCATTTGTTGGGCTATATGGTCTGTG  
 TTATTATGTGTTGGAAAACAATGTTGGTAACAACCCGATATACCAGACAC  
 1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA  
 GAACGTTTCGTTTATCACCTGTTTCATACCTATCTCCTGACATCGTCACTTT  
 1451 AGGCTGAACAACAGTGGGCTCTTTATGCAGATGGTTCAATACGTCCTCAG  
 TCCGACTTGTTGTACCCGAGAAAATACGTCACCAAGTTATGCAGGAGTC  
 1501 CAAAACCGAGATAATTGCCCTTACAAGTGATTCTAATATACGGGAAACAGT  
 GTTTTGGCTCTATTAAACGGAATGTTCACTAAGATTATATGCCCTTTGTCA  
 1551 TGTTAAGATCCTCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT  
 ACAATTCTAGGAGAGAACACCGGGACGTAGGAGACCGGTTGCTACCTACA  
 1601 TCAAGAATGATGGAACCATTTTAAATTTGTATAGTGGATTGGTGTAGAT  
 AGTTCCTTACTACCTTGGTAAAATTTAAACATATCACCTAACCACAATCTA  
 1651 GTGAGGCGATCGGATCCGAGCCTTAAACAAATCATTCTTTACCCTCTCCA  
 CACTCCGCTAGCCTAGGCTCGGAATTTGTTTGTAGTAAGAAATGGGAGAGGT  
 1701 TGGTGACCCAAACCAAATATGGTTACCATTATTTTGATAGACAGATTACT  
 ACCACTGGGTTTGGTTTATACCAATGGTAATAAACTATCTGTCTAATGA  
 1751 CTCTTGCAAGTGTGTGTGTCCTGCCATGAAAATAGATGGCTTAAATAAAAA  
 GAGAACGTCACACACACAGGACGGTACTTTTATCTACCGAATTTATTTTT  
 1801 GGACATTGTAAATTTTGTAACTGAAAGGACAGCAAGTTATATCGAATTCC  
 CCTGTAACATTTAAACATTGACTTTCCTGTCGTTCAATATAGCTTAAGG  
 1851 TGCAG  
 ACGTC

**FIGURE 11A**

**FIGURE 11B**

WT preporicin linker

primer MAL-E1

5' - AATAATTCACAGCATCAGGCTGATGTTGTATG -3'

TCTTTGCTTATAAGGCCAGTGGTGCCAAATTTTAAT  
AGAAACGAATATTCGGTCACCCAGGTTTAAATTA

3' - GGTAGCAGTGTCAAATTTAAGGTTCTATACGAT -5'

primer MAL-E2

PCR mutagenesis

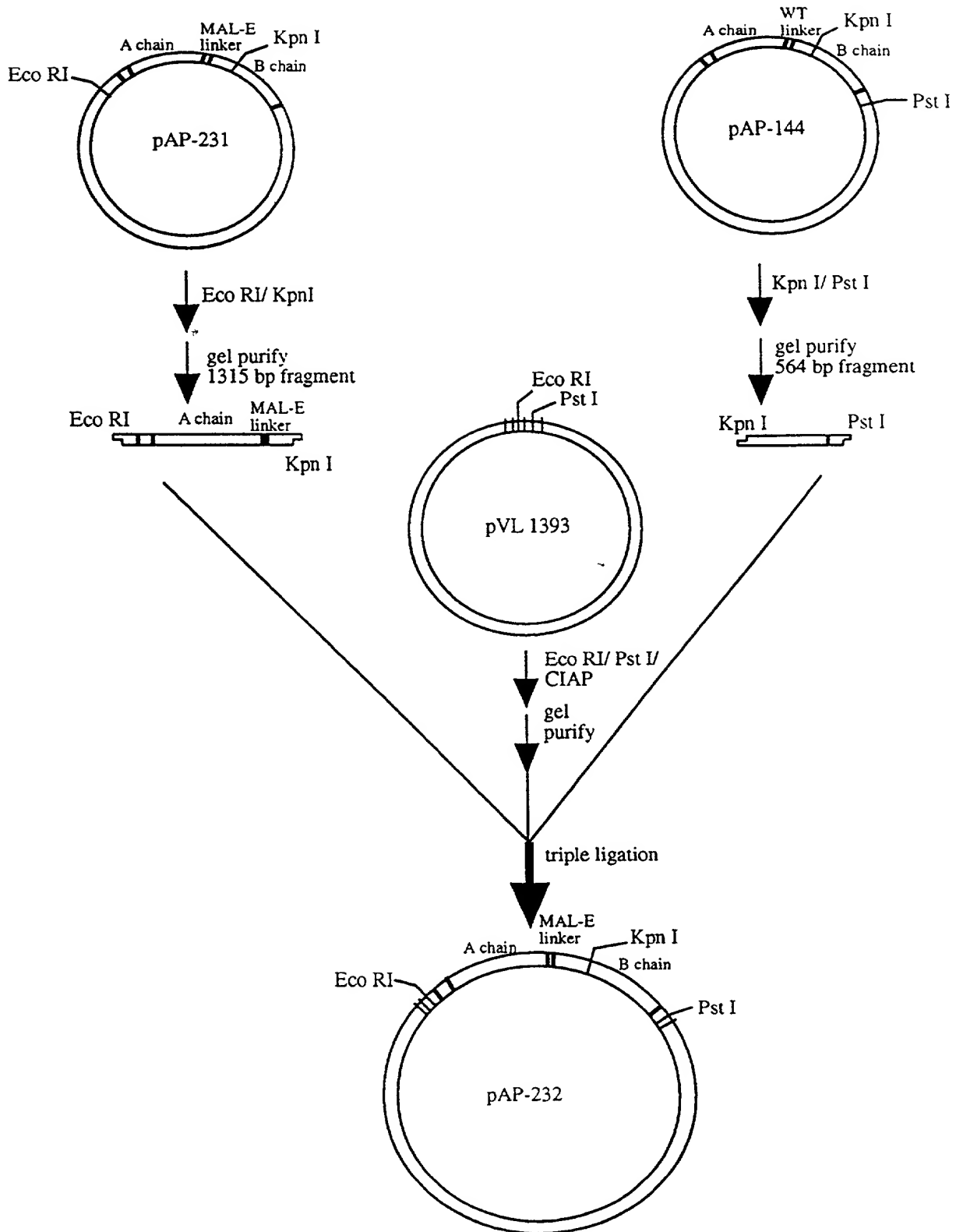
ligate with pBluescript SK

pAP 231 linker  
(MAL-E variant)

AAATTCAGATATGCTAAATAATTCACAGCATCAG  
TTTAAGGTTCTATACGATTATTAAGTGTGCTAGTC

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**FIGURE 11C**



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**FIGURE 11D**

10 20 30 40 50

1 GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT  
CTTAAGTACTTTGGCCCTCCTTTATGATAACATTATACCTACATACGTCA

51 GGCAACATGGCTTTGTTTTGGATCCACCTCAGGGTGGTCTTTCACATTAG  
CCGTTGTACCGAAACAAACCTAGGTGGAGTCCCACCAGAAAGTGTAATC

101 AGGATAACAACATATTCCTCCAAACAATACCCAATTATAAACTTTACCACA  
TCCTATTGTTGTATAAGGGGTTTGTATGGGTTAATATTTGAAATGGTGT

151 GCGGGTGCCACTGTGCAAAGCTACACAACTTTATCAGAGCTGTTTCGCGG  
CGCCCCACGGTGACACGTTTCGATGTGTTTGAAATAGTCTCGACAAGCGCC

201 TCGTTTAACAACCTGGAGCTGATGTGAGACATGATATACCAGTGTGCCAA  
AGCAAATTGTTGACCTCGACTACACTCTGTACTATATGGTCACAACGGTT

251 ACAGAGTTGGTTTGCCTATAAACCAACGGTTTATTTTAGTTGAACTCTCA  
TGTCTCAACCAAACGGATATTTGGTTGCCAAATAAAATCAACTTGAGAGT

301 AATCATGCAGAGCTTTCTGTTACATTAGCGCTGGATGTCACCAATGCATA  
TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT

351 TGTGGTCGGCTACCGTGCTGGAATAGCGCATATTTCTTTTCATCCTGACA  
ACACCAGCCGATGGCAGCACCTTTATCGCGTATAAAGAAAGTAGGACTGT

401 ATCAGGAAGATGCAGAAGCAATCACTCATCTTTTCACTGATGTTCAAAAT  
TAGTCCTTCTACGTCTTCGTTAGTGAGTAGAAAAGTGAATAAAGTTTTA

451 CGATATACATTTCGCCTTTGGTGGTAATTATGATAGACTTGAACAACTTGC  
GCTATATGTAAGCGGAAACCACCATTAACTATCTGAACCTGTTGAACG

501 TGGTAATCTGAGAGAAAATATCGAGTTGGGAAATGGTCCACTAGAGGAGG  
ACCATTAGACTCTCTTTTATAGCTCAACCTTTACCAGGTGATCTCCTCC

551 CTATCTCAGCGCTTTATTATTACAGTACTGGTGGCACTCAGCTTCCAACCT  
GATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA

601 CTGGCTCGTTTCCTTTATAATTTGCATCCAAATGATTTTCAAGAGCAGCAAG  
GACCGAGCAAGGAAATATTAAACGTAGGTTTACTAAAGTCTTCGTCGTTT

651 ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA  
TAAGGTTATATAACTCCCTCTTTACGCGTGCTCTTAATCCATGTTGGCCT

701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAAATAGTTGGGGGAGA  
CTAGACGTGGTCTAGGATCGCATTAATGTGAACTCTTATCAACCCCTCT

751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTTGCTAGTCCAAT  
GAAAGGTGACGTTAAGTTCTCAGATTGGTTCTCGGAAACGATCAGGTTA

801 TCAACTGCAAAGACGTAATGGTTCCAAATTCAGTGTGTACGATGTGAGTA  
AGTTGACGTTTCTGCATTACCAAGGTTTAAAGTCACACATGCTACACTCAT

851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGCACCTCCACCA  
ATAATTAGGGATAGTATCGAGAGTACCACATATCTACGCGTGGAGGTGGT

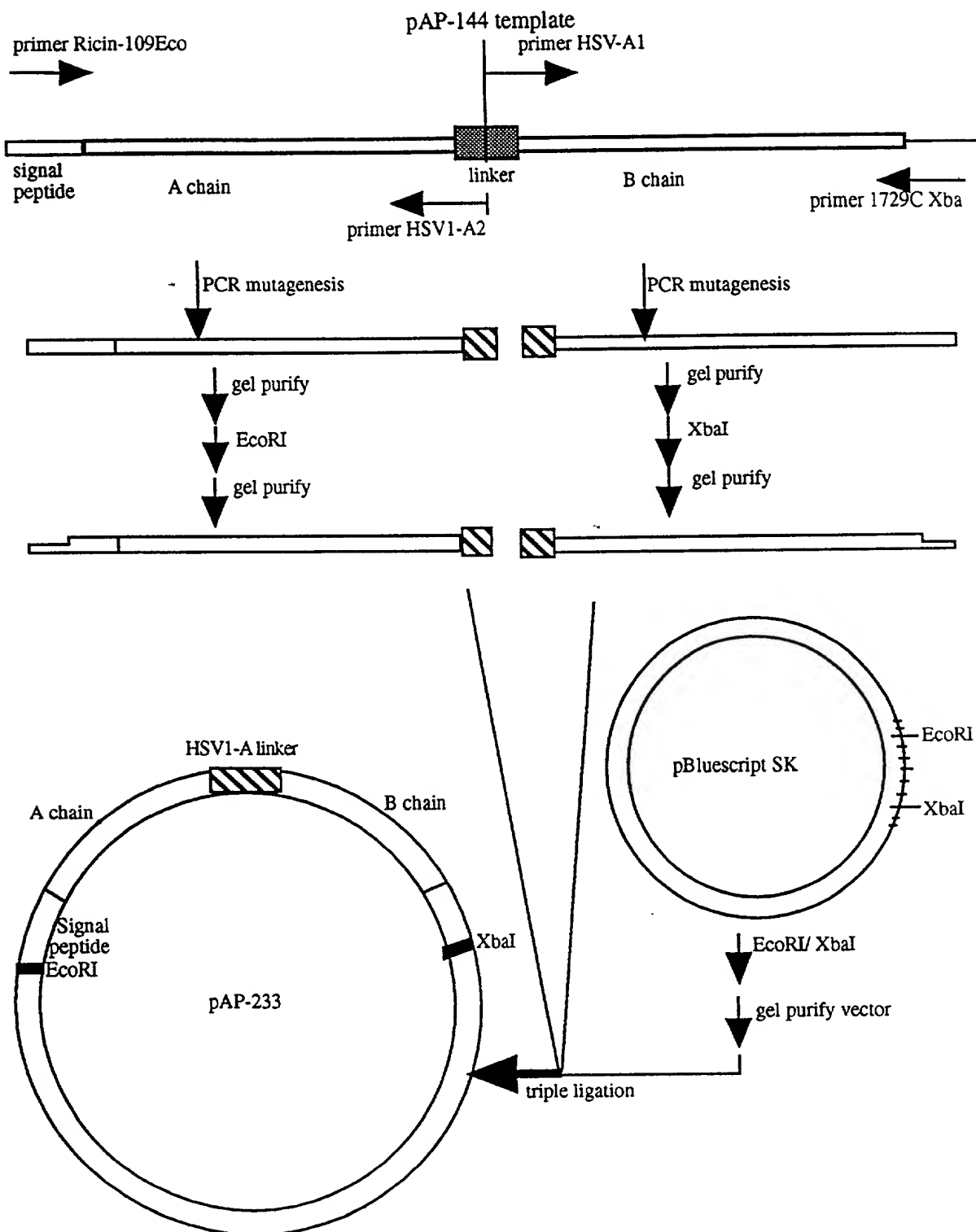
901 TCGTCACAGTTTAAATTCCAAGATATGCTAAATAATTCACAGCATCAGGC  
AGCAGTGTCAAATTTAAGGTTCTATACGATTTATTAAGTGTGCTAGTCCG

095451-044400



**FIGURE 11D (CONT'D)**

951 TGATGTTTGTATGGATCCTGAGCCCATAGTGCCTATCGTAGGTCGAAATG  
 ACTACAAACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTTAC  
 1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAACGCAATA  
 CAGATACACAACACTACAATCCCTACCTTCTAAGGTGTTGCCTTTGCGTTAT  
 1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT  
 GTCAACACCGGTACGTTTACGATTATGTCTACGTTTAGTCGAGACCTGAAA  
 1101 GAAAAGAGACAATACTATTTCGATCTAATGGAAAGTGTTTAACTACTTACG  
 CTTTTCTCTGTTATGATAAGCTAGATTACCTTTCACAAATTGATGAATGC  
 1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA  
 CCATGTCAGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT  
 1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC  
 TGACTACGGTGGGCGACCGTTTATACCCTATTACCTTGGTAGTATTTAGG  
 1251 CAGATCTAGTCTAGTTTTAGCAGCGACATCAGGGAACAGTGGTACCACAC  
 GTCTAGATCAGATCAAAATCGTCGCTGTAGTCCCTTGTCAACCATGGTGTG  
 1301 TTACAGTGCAAACCAACATTTATGCCGTTAGTCAAGGTTGGCTTCTACT  
 AATGTCACGTTTGGTTGTAAATACGGCAATCAGTTCCAACCGAAGGATGA  
 1351 AATAATACACAACCTTTTGTACAACCATTTGTTGGGCTATATGGTCTGTG  
 TTATTATGTGTTGGAAAACAATGTTGGTAACAACCCGATATACCAGACAC  
 1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA  
 GAACGTTTCGTTTATCACCTGTTTCATACCTATCTCCTGACATCGTCACTTT  
 1451 AGGCTGAACAACAGTGGGCTCTTTATGCAGATGGTTCAATACGTCCTCAG  
 TCCGACTTGTTGTCACCCGAGAAATACGTCTACCAAGTTATGCAGGAGTC  
 1501 CAAAACCGAGATAATTGCCTTACAAGTGATTCTAATATACGGGAAACAGT  
 GTTTTGGCTCTATTAAACGGAATGTTCACTAAGATTATATGCCCTTTGTCA  
 1551 TGTTAAGATCCTCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT  
 ACAATTCTAGGAGAGAAACACCGGGACGTAGGAGACCGGTTGCTACCTACA  
 1601 TCAAGAATGATGGAACCATTTTAAATTTGTATAGTGGATTGGTGTAGAT  
 AGTTCTTACTACCTTGGTAAATTTAAACATATCACCTAACCAACAATCTA  
 1651 GTGAGGCGATCGGATCCGAGCCTTAAACAAATCATTCCTTTACCCTCTCCA  
 CACTCCGCTAGCCTAGGCTCGGAATTTGTTTAGTAAGAAATGGGAGAGGT  
 1701 TGGTGACCCAAACCAATATGGTTACCATTATTTTGATAGACAGATTACT  
 ACCACTGGGTTTGGTTTATACCAATGGTAATAAACTATCTGTCTAATGA  
 1751 CTCTTGCAAGTGTGTGTGTCCTGCCATGAAAATAGATGGCTTAAATAAAAA  
 GAGAACGTCACACACACAGGACGGTACTTTTATCTACCGAATTTATTTTT  
 1801 GGACATTGTAAATTTTGTAACTGAAAGGACAGCAAGTTATATCGAATTCC  
 CCTGTAACATTTAAACATTGACTTTCTCTGTCGTTCAATATAGCTTAAGG  
 1851 TGCAG  
 ACGTC

**FIGURE 12A**

## FIGURE 12B

WT preprorin linker

primer HSV1-A

5' - TCGTCGGGCACATGTTAATGCTGATGTTTGT -3'

TCTTTGCTTATAAGGCCAGTGGTGCCAAATTTTAAT  
AGAAACGAATATTCCGGT CACCACGGTTAAAAATTA

3' - AGCAGTGTCAAAAAGACGCGAACATTTGCGT-5'

primer HSV1-A

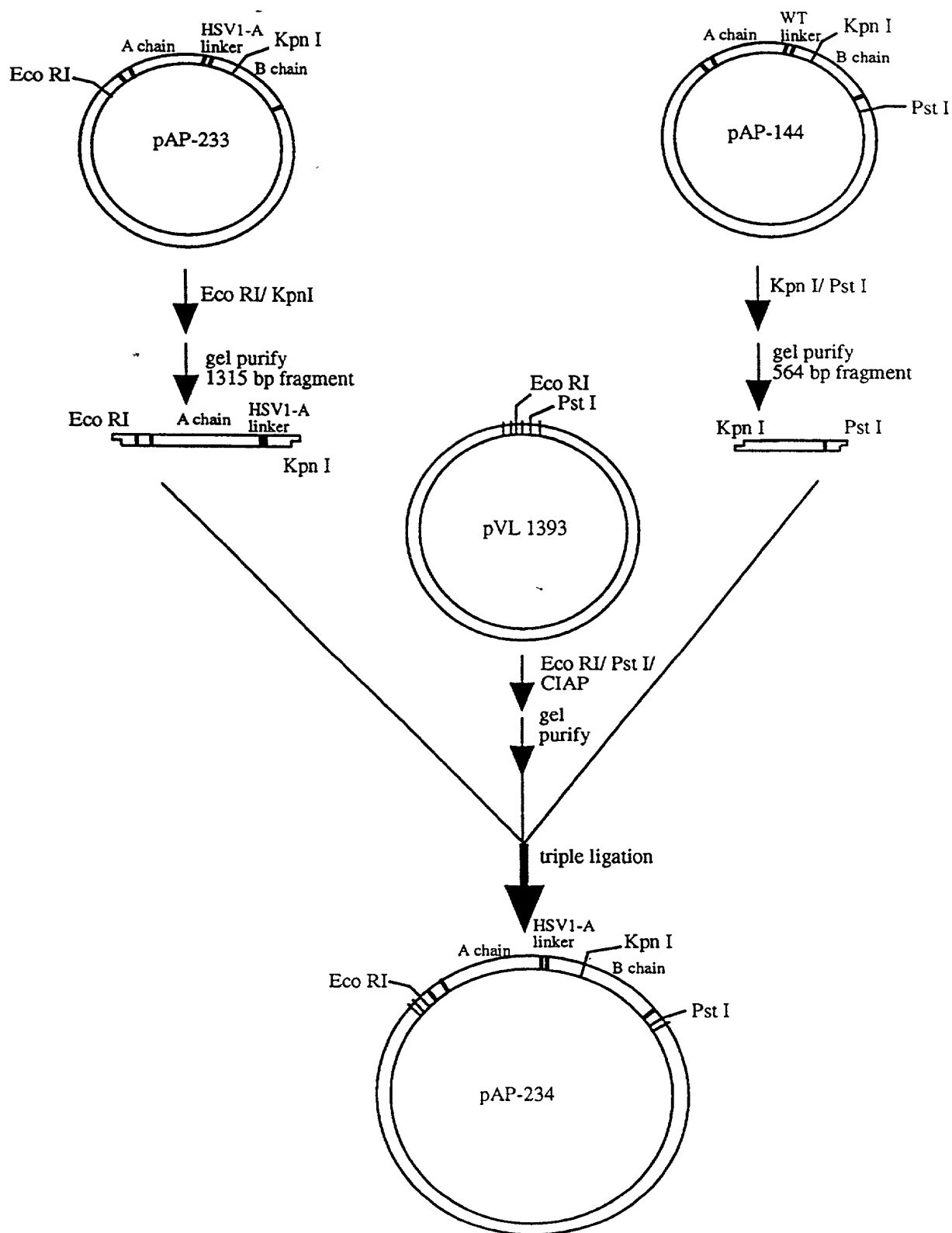
PCR mutagenesis  
ligate with pBluescript SK

pAP 233 linker  
(HSV1-A variant)

TCTGCGCTTGTAACGCATCGTCGGCACATGTTAAT  
AGACGGAACATTTGCGTAGCAGCCGGTACAATTA

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**FIGURE 12C**



004440 " 454450

**FIGURE 12D**

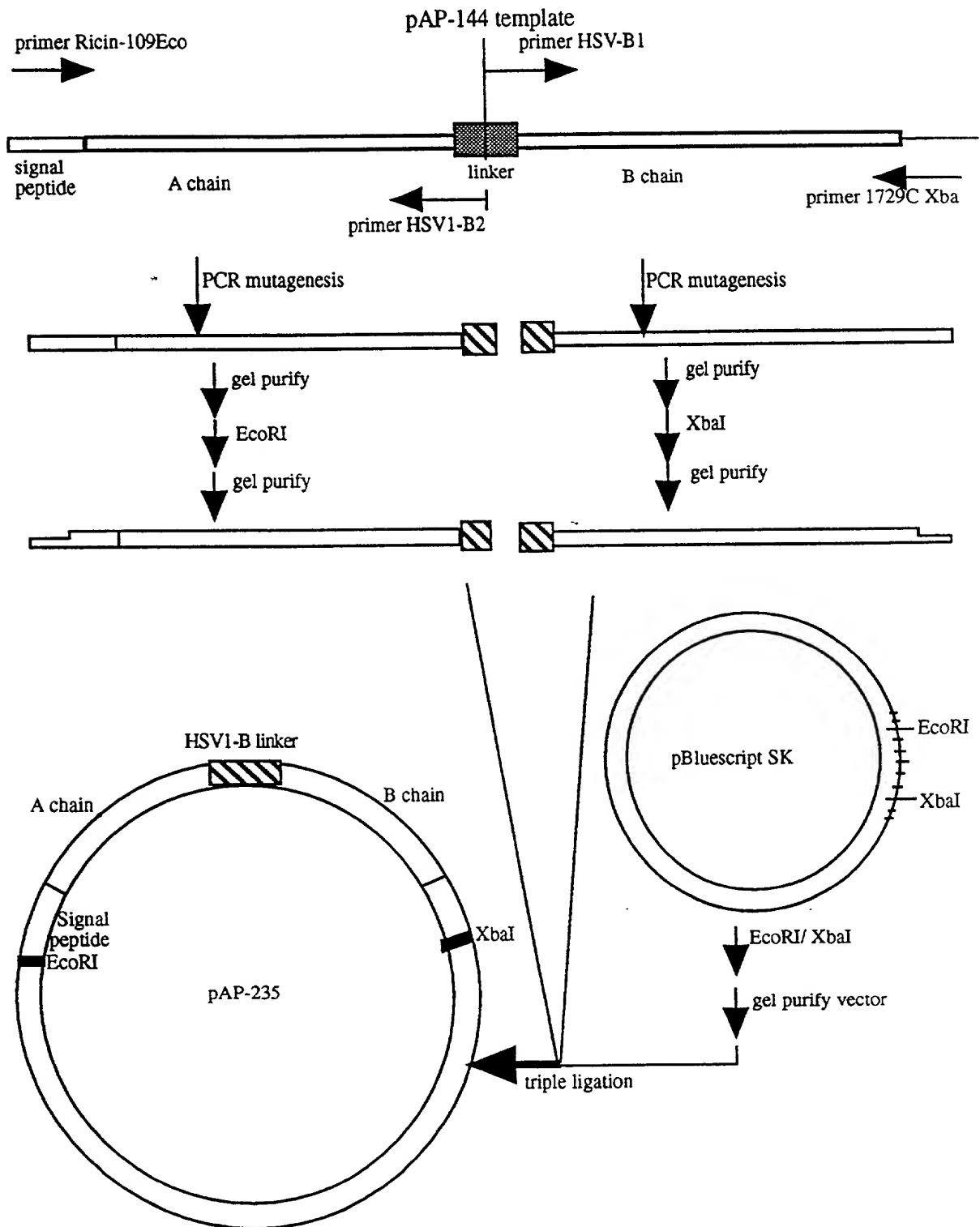
10            20            30            40            50  
 1 GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT  
 CTTAAGTACTTTGGCCCTCCTTTATGATAACATTATACCTACATACGTCA  
 51 GGCAACATGGCTTTGTTTTGGATCCACCTCAGGGTGGTCTTTCACATTAG  
 CCGTTGTACCGAAACAAAACCTAGGTGGAGTCCCACCAGAAAGTGTAATC  
 101 AGGATAACAACATATTCCCCAAACAATACCCAATTATAAACTTTACCACA  
 TCCTATTGTTGTATAAGGGGTTTGTATGGGTAAATATTTGAAATGGTGT  
 151 GCGGGTGCCACTGTGCAAAGCTACACAAACTTTATCAGAGCTGTTTCGCGG  
 CGCCCACGGTGACACGTTTCGATGTGTTTGAAATAGTCTCGACAAGCGCC  
 201 TCGTTTAACAACTGGAGCTGATGTGAGACATGATATACCAGTGTGCCAA  
 AGCAAATGTTGACCTCGACTACACTCTGTACTATATGGTCACAACGGTT  
 251 ACAGAGTTGGTTTGCCTATAAACCACGGTTTATTTTAGTTGAACTCTCA  
 TGTCTCAACCAACGGATATTTGGTTGCCAAATAAAATCAACTTGAGAGT  
 301 AATCATGCAGAGCTTCTGTTACATTAGCGCTGGATGTCACCAATGCATA  
 TTAGTACGTCTCGAAAGACAAATGTAATCGCGACCTACAGTGGTTACGTAT  
 351 TGTGGTCGGCTACCGTGTGGAATAGCGCATATTTCTTTCATCCTGACA  
 ACACCAGCCGATGGCAGGACCTTTATCGCGTATAAAGAAAGTAGGACTGT  
 401 ATCAGGAAGATGCAGAAGCAATCACTCATCTTTTCACTGATGTTCAAAAT  
 TAGTCCCTTCTACGTCTTCGTTAGTGAGTAGAAAAGTGAAGTACAAGTTTGA  
 451 CGATATACATTTCGCCCTTTGGTGGTAATTATGATAGACTTGAACAACCTGC  
 GCTATATGTAAGCGGAAACCACCATTAACTATCTGAACCTGTTGAACG  
 501 TGGTAATCTGAGAGAAAATATCGAGTTGGGAAATGGTCCACTAGAGGAGG  
 ACCATTAGACTCTCTTTTATAGCTCAACCTTTACCAGGTGATCTCCTCC  
 551 CTATCTCAGCGCTTTATTATTACAGTACTGGTGGCACTCAGCTTCCAAC  
 GATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA  
 601 CTGGCTCGTTCCTTTATAATTTGCATCCAAATGATTTCAGAAGCAGCAAG  
 GACCGAGCAAGGAAATATTAACGTAGGTTTACTAAAGTCTTCGTCGTTT  
 651 ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA  
 TAAGGTTATATAACCCCTCTTTACGCGTGCTCTTAATCCATGTTGGCCT  
 701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGGAGA  
 CTAGACGTGGTCTAGGATCGCATTAATGTGAACCTTATCAACCCCTCT  
 751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTTGCTAGTCCAAT  
 GAAAGGTGACGTTAAGTTCTCAGATTGGTTCCCTCGGAAACGATCAGGTTA  
 801 TCAACTGCAAAGACGTAATGGTTCCAAATTCAGTGTGTACGATGTGAGTA  
 AGTTGACGTTTCTGCATTACCAAGGTTTAAGTCACACATGCTACACTCAT  
 851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGCACCTCCACCA  
 ATAATTAGGGATAGTATCGAGAGTACCACATATCTACGCGTGGAGGTGGT  
 901 TCGTCACAGTTTTCTGCGCTTGTAACGCATCGTCGGCACATGTTAATGC  
 AGCAGTGTCAAAGACGCGAACATTTGCGTAGCAGCCGTGTACAATTACG

**FIGURE 12D (CONT'D)**

951 TGATGTTTGTATGGATCCTGAGCCCATAGTGCGTATCGTAGGTCGAAATG  
 ACTACAAACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTTAC  
 1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA  
 CAGATACACAACCTACAATCCCTACCTTCTAAGGTGTTGCCTTTGCGTTAT  
 1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT  
 GTCAACACCGGTACGTTTACAGATTATGTCTACGTTTAGTCGAGACCTGAAA  
 1101 GAAAAGAGACAATACTATTTCGATCTAATGGAAAGTGTTAACTACTTACG  
 CTTTTCTCTGTTATGATAAGCTAGATTACCTTTCACAAATTGATGAATGC  
 1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA  
 CCATGTCAGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT  
 1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC  
 TGACTACGGTGGGCGACCGTTTATACCCTATTACCTTGGTAGTATTTAGG  
 1251 CAGATCTAGTCTAGTTTTAGCAGCGACATCAGGGAACAGTGGTACCACAC  
 GTCTAGATCAGATCAAAATCGTCGCTGTAGTCCCTTGTACCATGGTGTG  
 1301 TTACAGTGCAAACCAACATTTATGCCGTTAGTCAAGGTTGGCTTCCTACT  
 AATGTCACGTTTGGTTGTAAATACGGCAATCAGTTCCAACCGAAGGATGA  
 1351 AATAATACACAACCTTTTGTGTTACAACCATTTGTTGGGCTATATGGTCTGTG  
 TTATTTATGTGTTGGAAAACAAATGTTGGTAACAACCCGATATACCAGACAC  
 1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA  
 GAACGTTTCGTTTATCACCTGTTTCATACCTATCTGCTGACATCGTCACTTT  
 1451 AGGCTGAACAACAGTGGGCTCTTTATGCAGATGGTTCAATACGTCCTCAG  
 TCCGACTTGTGTTGCACCCGAGAAATACGTCACCAAGTTATGCAGGAGTC  
 1501 CAAAACCGAGATAATTGCCCTTACAAGTGATTCTAATATACGGGAAACAGT  
 GTTTTGGCTCTATTAACGGAATGTTTCACTAAGATTATATGCCCTTTGTCA  
 1551 TGTTAAGATCCTCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT  
 ACAATTCTAGGAGAGAACACCGGGACGTAGGAGACCGGTTGCTACCTACA  
 1601 TCAAGAATGATGGAACCATTTTAAATTTGTATAGTGGATTGGTGTAGAT  
 AGTTCTTACTACCTTGGTAAAATTTAAACATATCACCTAACCACAATCTA  
 1651 GTGAGGCGATCGGATCCGAGCCTTAAACAAATCATTTCTTTACCCTCTCCA  
 CACTCCGCTAGCCTAGGCTCGGAATTTGTTTGTAGTAAGAAATGGGAGAGGT  
 1701 TGGTGACCCAAACCAAATATGGTTACCATTATTTTGTATAGACAGATTACT  
 ACCACTGGGTTTGGTTTATACCAATGGTAATAAACTATCTGTCTAATGA  
 1751 CTCTTGCAAGTGTGTGTCTGCTGCCATGAAAATAGATGGCTTAAATAAAAA  
 GAGAACGTCACACACACAGGACGGTACTTTTATCTACCGAATTTATTTTT  
 1801 GGACATTGTAAATTTTGTAACTGAAAGGACAGCAAGTTATATCGAATTCC  
 CCTGTAACATTTAAACATTGACTTTCCTGTCGTTCAATATAGCTTAAGG  
 1851 TGCAG  
 ACGTC

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**FIGURE 13A**



## FIGURE 13B

WT preprorin linker

primer HSV1-B

5' - TCGGAGAAATTTAAGAAATGCTGATGTTTGT -3'

TCTTTGCTTATAAGGCCAGTGGTGCCAAATTTTAAT  
AGAAACGAATATTCGGTCACCCACGGTTTAAATTA  
3' - AGCAGTGTCAAAGATGCATAAATGTCCGT-5'

primer HSV1-B

PCR mutagenesis  
ligate with pBluescript SK

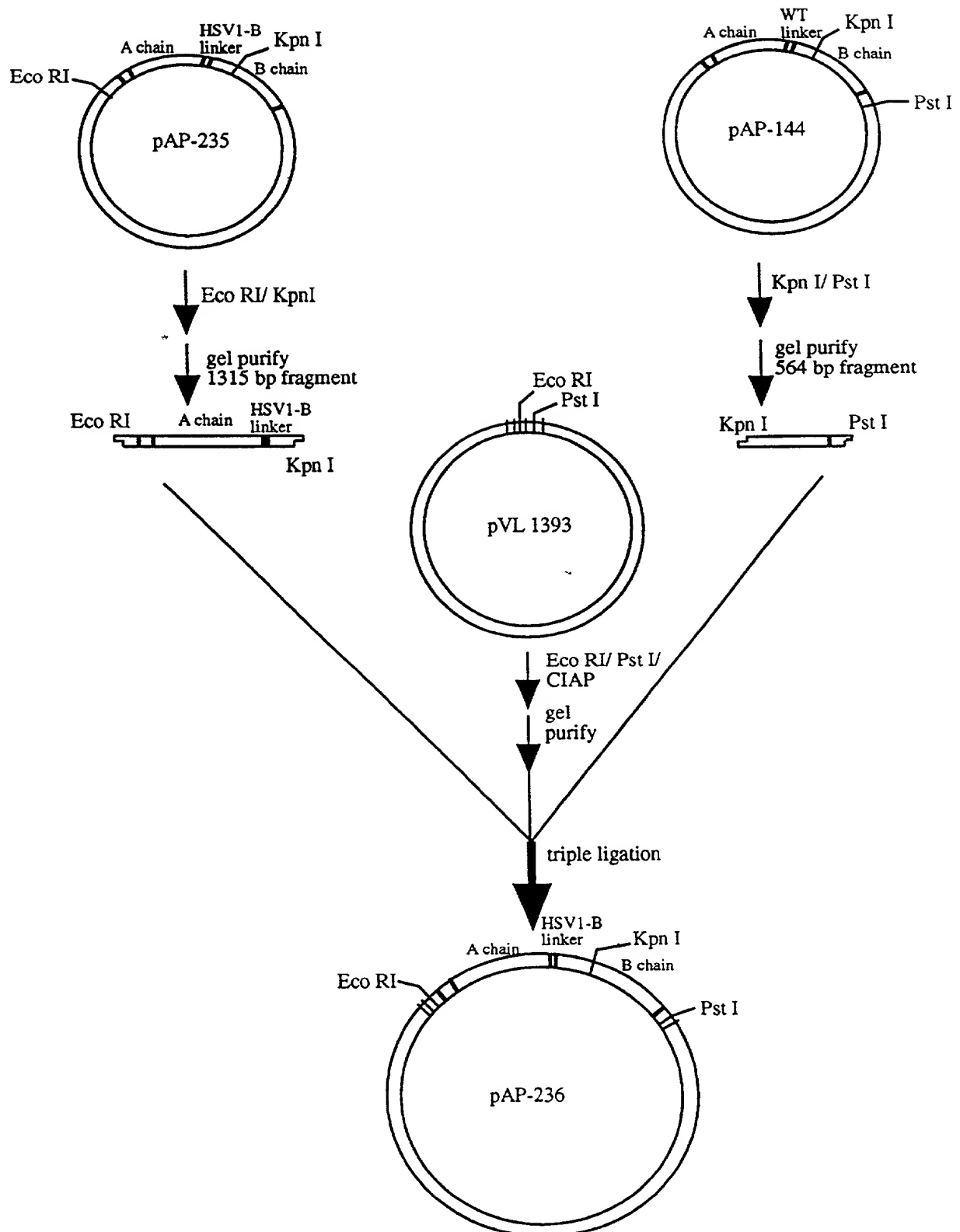
pAP 235 linker  
(HSV1-B variant)

TCTACGTATTACAGGCATCGGAGAAATTTAAGAAAT  
AGATGCATAAATGTCCGTAGCCTCTTAAATCTTA



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**FIGURE 13C**



004404 " 454550

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## FIGURE 13D

10 20 30 40 50  
1 GAATTCATGAAACCGGGAGGAAATAC'TATTGTAATATGGATGTATGCAGT  
CTTAAGTACTTTGGCCCTCCTTTATGATAACATTATACCTACATACGTCA  
51 GGCAACATGGCTTTGTTTTGGATCCACCTCAGGGTGGTCTTTCACATTAG  
CCGTTGTACCAGAAACAAACCTAGGTGGAGTCCCACCAGAAAGTGTAAATC  
101 AGGATAACAACATATTCCCCAAACAATACCCAATTATAAACTTTACCACA  
TCCTATTGTTGTATAAGGGGTTTGTATGGGTAAATATTTGAAATGGTGT  
151 GCGGGTGCCACTGTGCAAAGCTACACAACTTTATCAGAGCTGTTTCGCGG  
CGCCACGGTGACACGTTTCGATGTGTTTGAAATAGTCTCGACAAGCGCC  
201 TCGTTTAACAACTGGAGCTGATGTGAGACATGATATACCAGTGTTGCCAA  
AGCAAATTGTTGACCTCGACTACACTCTGTACTATATGGTCACAACGGTT  
251 ACAGAGTTGGTTTGCCTATAAACCAACGGTTTATTTTAGTTGAACTCTCA  
TGTCTCAACCAAACGGATATTTGGTTGCCAAATAAAATCAACTTGAGAGT  
301 AATCATGCAGAGCTTTCTGTTACATTAGCGCTGGATGTCACCAATGCATA  
TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT  
351 TGTGGTCGGCTACCGTGCTGGAAATAGCGCATATTTCTTTTCATCCTGACA  
ACACCAGCCGATGGCAGACCTTTATCGCGTATAAAGAAAGTAGGACTGT  
401 ATCAGGAAGATGCAGAAGCAATCACTCATCTTTTCACTGATGTTCAAAAT  
TAGTCCTTCTACGTCTTCGTTAGTGAGTAGAAAAGTGAAGTACAAGTTTGA  
451 CGATATACATTTCGCCTTTGGTGGTAATTATGATAGACTTGAACAACTTGC  
GCTATATGTAAGCGGAAACCACCATTAATACTATCTGAAGTTGTTGAACG  
501 TGGTAATCTGAGAGAAAATATCGAGTTGGGAAATGGTCCACTAGAGGAGG  
ACCATTAGACTCTCTTTTATAGCTCAACCTTTACCAGGTGATCTCCTCC  
551 CTATCTCAGCGCTTTATTATTACAGTACTGGTGGCACTCAGCTTCCAAC  
GATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA  
601 CTGGCTCGTTCCTTTATAATTTGCATCCAAATGATTTTCAGAAGCAGCAAG  
GACCGAGCAAGGAAATATTAAACGTAGGTTTACTAAAGTCTTCGTCGTTC  
651 ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA  
TAAGGTTATATAACTCCCTCTTTACGCGTGCTCTTAATCCATGTTGGCCT  
701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGGAGA  
CTAGACGTGGTCTAGGATCGCATTAATGTGAAGTCTTATCAACCCCTCT  
751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTTGCTAGTCCAAT  
GAAAGGTGACGTTAAGTTCTCAGATTGGTTCTCGGAAACGATCAGGTTA  
801 TCAACTGCAAAGACGTAATGGTTCCAAATTCAGTGTGTACGATGTGAGTA  
AGTTGACGTTTCTGCATTACCAAGGTTTAAAGTCACACATGCTACACTCAT  
851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGCACCTCCACCA  
ATAATTAGGGATAGTATCGAGAGTACCACATATCTACGCGTGGAGGTGGT  
901 TCGTCACAGTTTTCTACGTATTTACAGGCATCGGAGAAATTTAAGAATGC  
AGCAGTGTCAAAGATGCATAAATGTCCGTAGCCTCTTTAAATCTTACG

**FIGURE 13D (CONT'D)**

951 TGATGTTTGTATGGATCCTGAGCCCATAGTGCGTATCGTAGGTCGAAATG  
ACTACAAACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTTAC

1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA  
CAGATACACAAC TACAATCCCTACCTTCTAAGGTGTTGCCTTTGCGTTAT

1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT  
GTCAACACCGGTACGTTTACAGATTATGTCTACGTTTAGTCGAGACCTGAAA

1101 GAAAAGAGACAATACTATTTCGATCTAATGGAAAGTGTTTAACTACTTACG  
CTTTTCTCTGTTATGATAAGCTAGATTACCTTTCACAAATTGATGAATGC

1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA  
CCATGTACAGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT

1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC  
TGACTACGGTGGGCGACCGTTTATACCCTATTACCTTGGTAGTATTTAGG

1251 CAGATCTAGTCTAGTTTTAGCAGCGACATCAGGGAACAGTGGTACCACAC  
GTCTAGATCAGATCAAAATCGTCGCTGTAGTCCCTTGTCACCATGGTGTG

1301 TTACAGTGCAAACCAACATTTATGCCGTTAGTCAAGGTTGGCTTCCTACT  
AATGTCACGTTTGGTTGTAAATACGGCAATCAGTTCCAACCGAAGGATGA

1351 AATAATACACAACCTTTTGTACAACCATTTGTTGGGCTATATGGTCTGTG  
TTATTATGTGTTGGAACAATGTTGGTAACAACCCGATATACCAGACAC

1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA  
GAACGTTTCGTTTATCACCTGTTTATACCTATCTCCTGACATCGTCACTTT

1451 AGGCTGAACAACAGTGGGCTCTTTATGCAGATGGTTCAATACGTCTCAG  
TCCGACTTGTTGTCACCCGAGAAATACGTCTACCAAGTTATGCAGGAGTC

1501 CAAAACCGAGATAATTGCCTTACAAGTGATTCTAATATACGGGAAACAGT  
GTTTTGGCTCTATTAAACGGAATGTTCACTAAGATTATATGCCCTTTGTCA

1551 TGTTAAGATCCTCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT  
ACAATTCTAGGAGAGAAACACCGGGACGTAGGAGACCGGTTGCTACCTACA

1601 TCAAGAATGATGGAACCATTTTAAATTTGTATAGTGGATTGGTGTTAGAT  
AGTTCTTACTACCTTGGTAAAATTTAAACATATCACCTAACCACAATCTA

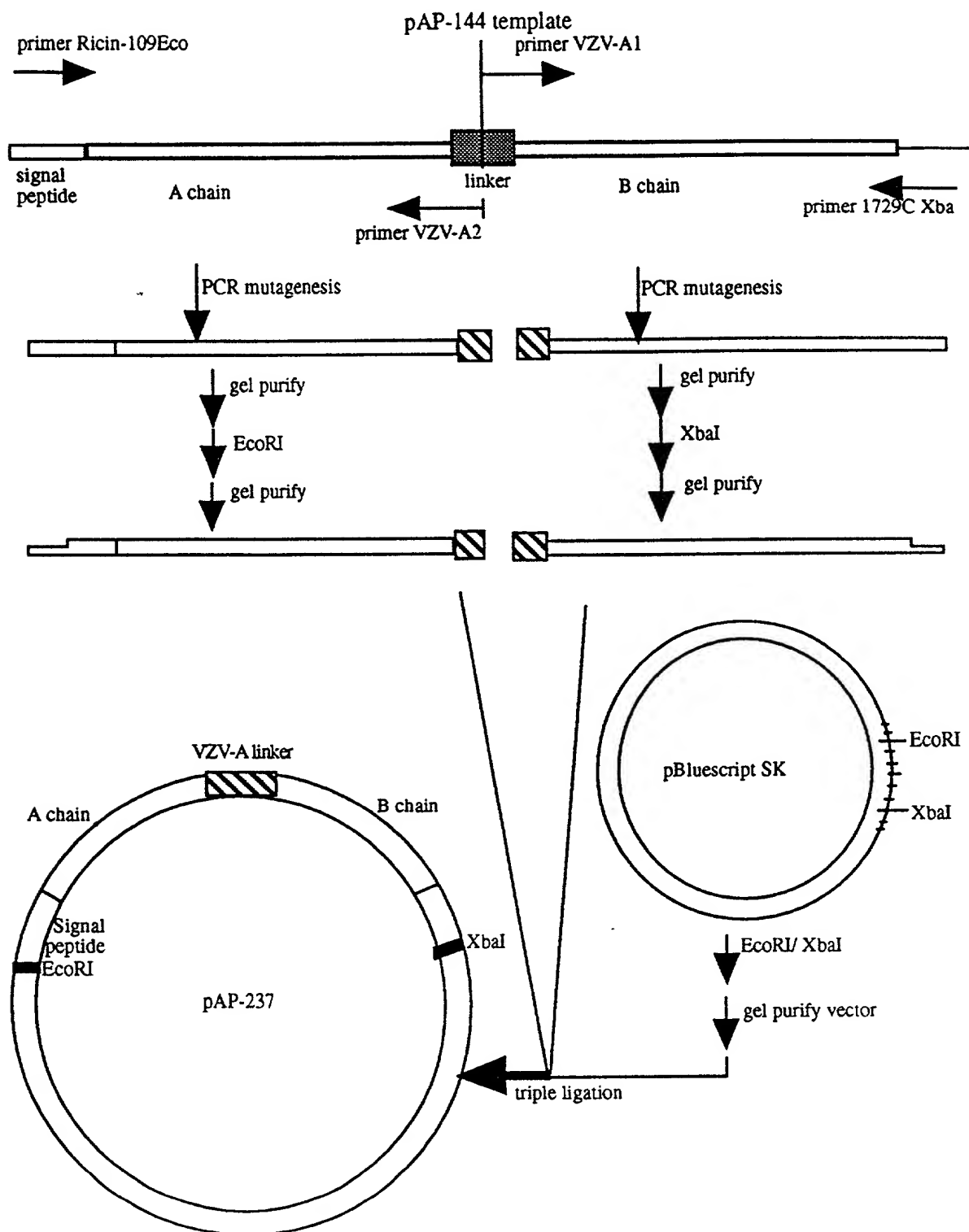
1651 GTGAGGCGATCGGATCCGAGCCTTAAACAAATCATTCCTTTACCCTCTCCA  
CACTCCGCTAGCCTAGGCTCGGAATTTGTTTAGTAAGAAATGGGAGAGGT

1701 TGGTGACCCAAACCAAATATGGTTACCATTATTTTGATAGACAGATTACT  
ACCACTGGGTTTGGTTTATACCAATGGTAATAAACTATCTGTCTAATGA

1751 CTCTTGCAGTGTGTGTGTCCTGCCATGAAAATAGATGGCTTAAATAAAAA  
GAGAACGTCACACACAGGACGGTACTTTTATCTACCGAATTTATTTTT

1801 GGACATTGTAAATTTTGTAACTGAAAGGACAGCAAGTTATATCGAATTCC  
CCTGTAACATTTAAACATTGACTTTCCTGTCGTTCAATATAGCTTAAGG

1851 TGCAG  
ACGTC

**FIGURE 14A**

**FIGURE 14B**

**WT preproricin linker**

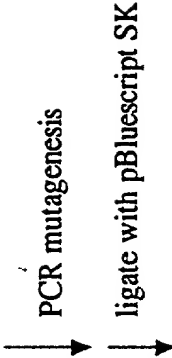
primer VZV-A1

5' - GTGGAGGCAAGTTCTTAATGCTGATGTTTGT -3'

TCCTTGCTTATAAGGCCAGTGGTGCCAAATTTTAAT  
AGAAACGAATATTCCTGGTCAACACGGTTTAAATTA

3' - AGCAGTGTCAAAAAGAGTCCCTACATTTGCGT-5'

primer VZV-A2

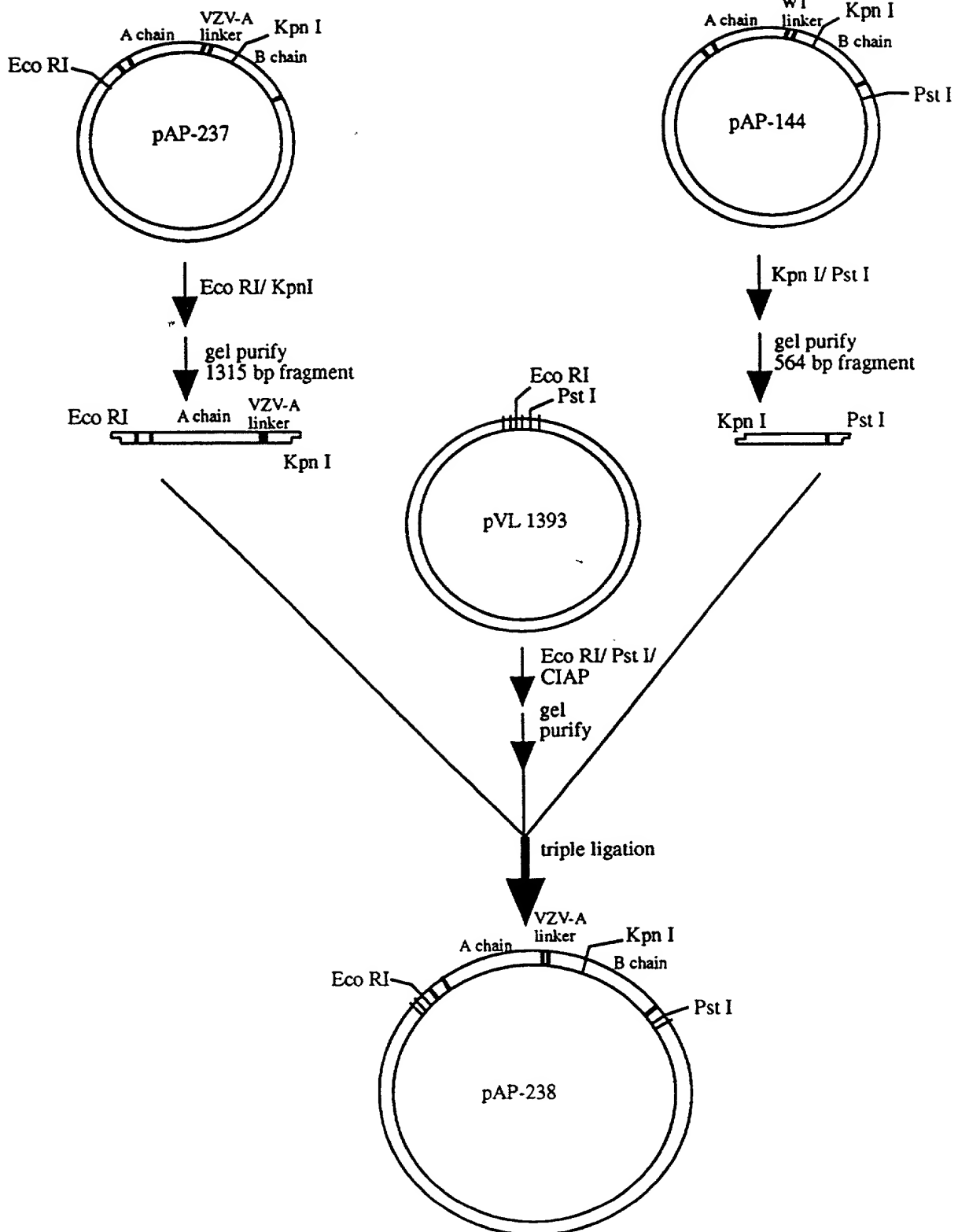


pAP 237 linker  
(VZV-A variant)

TCTCAGGATGTAAACGCAGTGGAGGCAAGTTCTAAT  
AGATCCTACATTTGCGTCACTCCGTTCAAGATTA

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**FIGURE 14C**



**FIGURE 14D**

10            20            30            40            50  
 1 GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT  
 CTTAAGTACTTTGGCCCTCCTTTATGATAACATTATACCTACATACGTCA  
 51 GGCAACATGGCCTTTGTTTTGGATCCACCTCAGGGTGGTCTTTCACATTAG  
 CCGTTGTACCGAAACAAACCTAGGTGGAGTCCCACCAGAAAGTGTAATC  
 101 AGGATAACAACATATTCCCCAAACAATACCCAAATTATAAACTTTACCACA  
 TCCTATTGTTGTATAAGGGGTTTGTATGGGTAAATATTTGAAATGGTGT  
 151 GCGGGTGCCACTGTGCAAAGCTACACAACTTTATCAGAGCTGTTTCGCGG  
 CGCCACGGTGACACGTTTCGATGTGTTTCAAATAGTCTCGACAAGCGCC  
 201 TCGTTTAACAACCTGGAGCTGATGTGAGACATGATATACCAGTGTGCCAA  
 AGCAAATTGTTGACCTCGACTACACTCTGTACTATATGGTCACAACGGTT  
 251 ACAGAGTTGGTTTGCCTATAAACCAACGGTTTATTTTAGTTGAACCTCTCA  
 TGTCTCAACCAAACGGATATTTGGTTGCCAAATAAAATCAACTTGAGAGT  
 301 AATCATGCAGAGCTTTCTGTACATTAGCGCTGGATGTCACCAATGCATA  
 TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT  
 351 TGTGGTCGGCTACCGTGCTGGAAATAGCGCATATTTCTTTCATCCTGACA  
 ACACCAGCCGATGGCAGCACCTTTATCGCGTATAAAGAAAGTAGGACTGT  
 401 ATCAGGAAGATGCAGAAGCAATCACTCATCTTTTCACTGATGTTCAAAT  
 TAGTCCTTCTACGTCTTCGTTAGTGAGTAGAAAAGTGAATAAGTTTGA  
 451 CGATATACATTTCGCCTTTGGTGGTAATTATGATAGACTTGAACAACCTGC  
 GCTATATGTAAGCGGAAACACCATTAACTATCTGAACTTGTGTAACG  
 501 TGGTAATCTGAGAGAAAATATCGAGTTGGGAAATGGTCCACTAGAGGAGG  
 ACCATTAGACTCTCTTTTATAGCTCAACCCTTTACCAGGTGATCTCCTCC  
 551 CTATCTCAGCGCTTTATTATTACAGTACTGGTGGCACTCAGCTTCCAAC  
 GATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA  
 601 CTGGCTCGTTCCTTTATAATTTGCATCCAAATGATTTTCAAGCAGCAAG  
 GACCGAGCAAGGAAATATTAAACGTAGGTTTACTAAAGTCTTCGTCGTTT  
 651 ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA  
 TAAGGTTATATAACTCCCTCTTTACGCGTGCTCTTAATCCATGTTGGCCT  
 701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGGAGA  
 CTAGACGTGGTCTAGGATCGCATTAATGTGAACTCTTATCAACCCCTCT  
 751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTTGCTAGTCCAAT  
 GAAAGGTGACGTTAAGTTCTCAGATTGGTTCCCTCGGAAACGATCAGGTTA  
 801 TCAACTGCAAAGACGTAATGGTTCCAAATTCAGTGTGTACGATGTGAGTA  
 AGTTGACGTTTCTGCATTACCAAGGTTTAAAGTCACACATGCTACACTCAT  
 851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGCACCTCCACCA  
 ATAATTAGGGATAGTATCGAGAGTACCACATATCTACGCGTGGAGGTGGT  
 901 TCGTCACAGTTTTTCTCAGGATGTAAACGCAGTGGAGGCAAGTTCTAATGC  
 AGCAGTGTCAAAGAGTCCTACATTTGCGTCACCTCCGTTCAAGATTACG

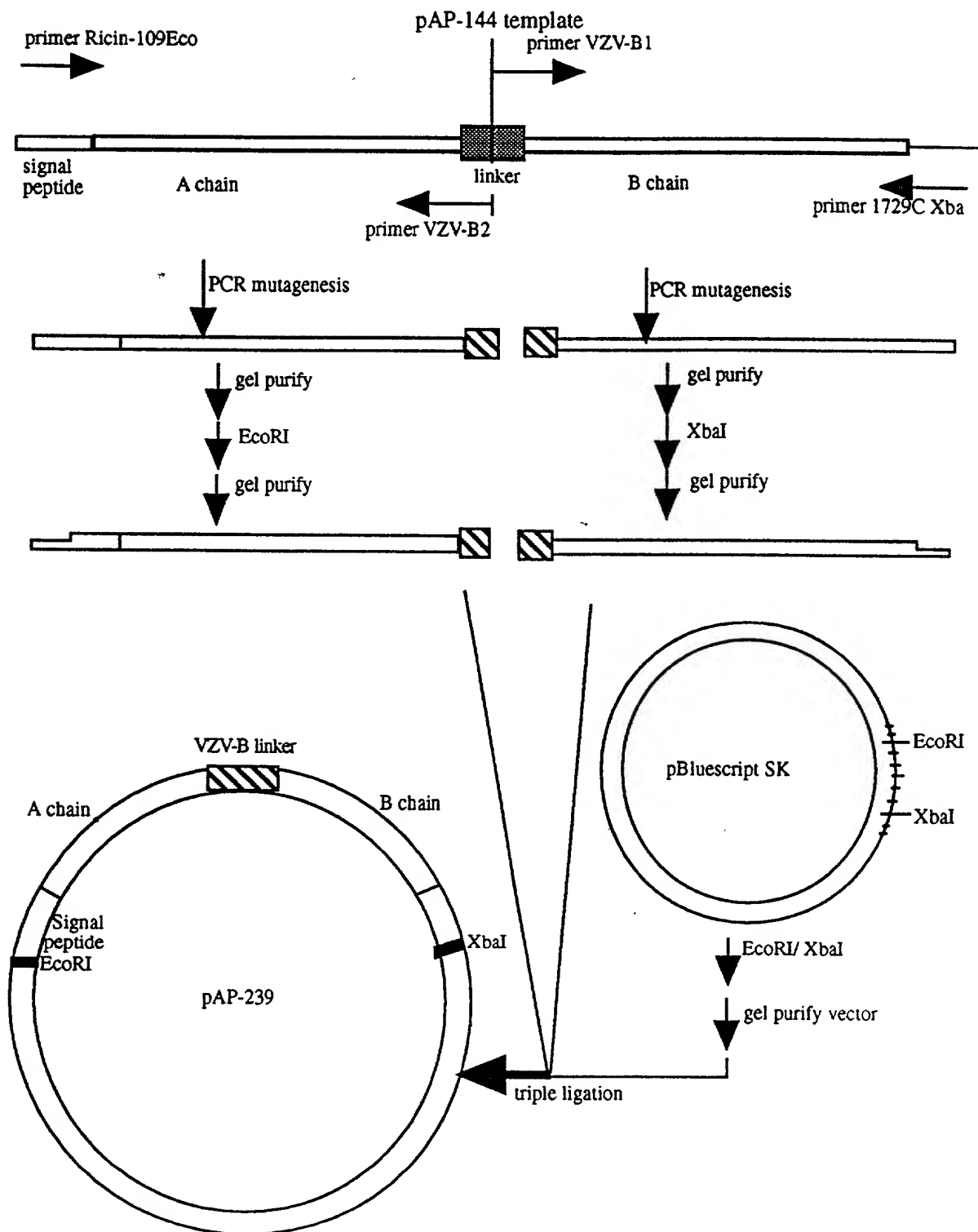
**FIGURE 14D (CONT'D)**

951 TGATGTTTGTATGGATCCTGAGCCCATAGTGCGTATCGTAGGTCGAAATG  
 ACTACAAACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTTAC  
 1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA  
 CAGATACACAACCTACAATCCCTACCTTCTAAGGTGTTGCCTTTGCGTTAT  
 1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT  
 GTCAACACCGGTACGTTAGATTATGTCTACGTTTAGTCGAGACCTGAAA  
 1101 GAAAAGAGACAATACTATTCGATCTAATGGAAAGTGTTTAACTACTTACG  
 CTTTTCTCTGTTATGATAAGCTAGATTACCTTTCACAAATTGATGAATGC  
 1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA  
 CCGTGTACAGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT  
 1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC  
 TGACTACGGTGGGCGACCGTTTATACCCTATTACCTTGGTAGTATTTAGG  
 1251 CAGATCTAGTCTAGTTTTAGCAGCGACATCAGGGAACAGTGGTACCACAC  
 GTCTAGATCAGATCAAAATCGTCGCTGTAGTCCCTTGTCAACCATGGTGTG  
 1301 TTACAGTGCAAACCAACATTTATGCCGTTAGTCAAGGTTGGCTTCCTACT  
 AATGTCACGTTTGGTTGTAAATACGGCAATCAGTTCCAACCGAAGGATGA  
 1351 AATAATACACAACCTTTTGTTACAACCATTGTTGGGCTATATGGTCTGTG  
 TTATTATGTGTTGGAAAACAATGTTGGTAACAACCCGATATACCAGACAC  
 1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA  
 GAACGTTTCGTTTATCACCTGTTTCATACCTATCTCCTGACATCGTCACTTT  
 1451 AGGCTGAACAACAGTGGGCTCTTTATGCAGATGGTTCAATACGTCCTCAG  
 TCCGACTTGTGTCAACCGAGAAATACGTCTACCAAGTTATGCAGGAGTC  
 1501 CAAAACCGAGATAATTGCCTTACAAGTGATTCTAATATACGGGAAACAGT  
 GTTTTGGCTCTATTAAACGGAATGTTCACTAAGATTATATGCCCTTTGTCA  
 1551 TGTTAAGATCCTCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT  
 ACAATTCTAGGAGAGAACACCGGGACGTAGGAGACCGGTTGCTACCTACA  
 1601 TCAAGAATGATGGAACCATTTTAAATTTGTATAGTGGATTGGTGTAGAT  
 AGTTCTTACTACCTTGGTAAAATTTAAACATATCACCTAACCAATCTA  
 1651 GTGAGGCGATCGGATCCGAGCCTTAAACAAATCATTCTTTACCCTCTCCA  
 CACTCCGCTAGCCTAGGCTCGGAATTTGTTTAGTAAGAAATGGGAGAGGT  
 1701 TGGTGACCCAAACCAAAATATGGTTACCATTATTTTGTAGACAGATTACT  
 ACCACTGGGTTTGGTTTATACCAATGGTAATAAACTATCTGTCTAATGA  
 1751 CTCTTGCAGTGTGTGTCTGCTGCCATGAAAAATAGATGGCTTAAATAAAAA  
 GAGAACGTCACACACACAGGACGGTACTTTTATCTACCGAATTTATTTTT  
 1801 GGACATTGTAAATTTTGTAACTGAAAGGACAGCAAGTTATATCGAATTCC  
 CCTGTAACATTTAAACATTGACTTTCCTGTCGTTCAATATAGCTTAAGG  
 1851 TGCAG  
 ACGTC



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**FIGURE 15A**



004440 "T5F560

# FIGURE 15B

WT preprorin linker

primer VZV-B1

5' - TCGACGGGATATGGTAATGCTGATGTTGT -3'

TCTTTGCTTATAAGGCCAGTGGTGCCAAATTTTAAT  
AGAAACGAATATTCCGGTCACCCACGGTTTAAATTA

3' - AGCAGTGTCAAAAGACACATAAAATGTCCGT-5'

primer VZV-A2

PCR mutagenesis

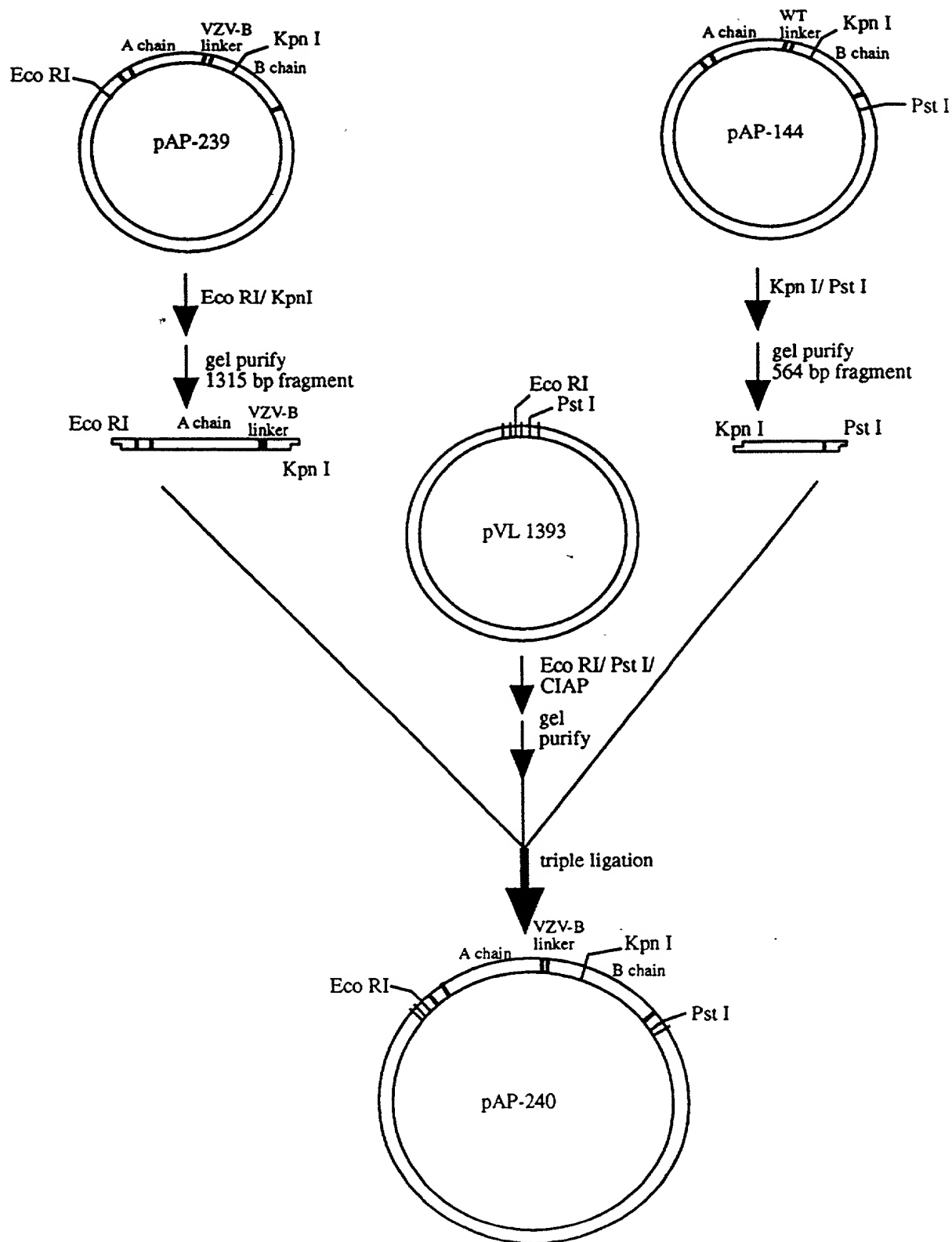
ligate with pBluescript SK

pAP 239 linker  
(VZV-B variant)

TCTGTGTATTACAGGCATCGACGGGATATGTAAT  
AGACACATAAATGTCCCGTAGCTGCCCTATACCATTA

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**FIGURE 15C**



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## FIGURE 15D

10 20 30 40 50

1 GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT  
CTTAAGTACTTTGGCCCTCCTTTATGATAACATTATACCTACATACGTCA

51 GGCAACATGGCTTTGTTTTGGATCCACCTCAGGGTGGTCTTTCACATTAG  
CCGTTGTACCGAAACAAACCTAGGTGGAGTCCCACCAGAAAGTGTAAATC

101 AGGATAACAACATATTCCCCAAACAATACCCAATTATAAACTTTACCACA  
TCCTATTGTTGTATAAGGGGTTTGTATTGGGTAAATATTTGAAATGGTGT

151 GCGGGTGCCACTGTGCAAAGCTACACAACTTTATCAGAGCTGTTTCGCGG  
CGCCACGGTGACACGTTTCGATGTGTTTGAAATAGTCTCGACAAGCGCC

201 TCGTTTAACAACTGGAGCTGATGTGAGACATGATATACCAAGTGTGCCAA  
AGCAAATTGTTGACCTCGACTACACTCTGTACTATATGGTCACAACGGTT

251 ACAGAGTTGGTTTGCCTATAAAACCAACGGTTTATTTTAGTTGAACTCTCA  
TGCTCAACCAAACGGATATTTGGTTGCCAAATAAAATCAACTTGAGAGT

301 AATCATGCAGAGCTTCTGTTACATTAGCGCTGGATGTCACCAATGCATA  
TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT

351 TGTGGTCGGCTACCGTGCTGGAAATAGCGCATATTTCTTTCATCCTGACA  
ACACCAGCCGATGGCAGCACCTTTATCGCGTATAAAGAAAGTAGGACTGT

401 ATCAGGAAGATGCAGAAGCAATCACTCATCTTTTCACTGATGTTCAAAAT  
TAGTCCCTCTACGTCTTCGTTAGTGAGTAGAAAAAGTGACTACAAGTTTTA

451 CGATATACATTTCGCTTTGGTGGTAATTATGATAGACTTGAACAACTTGC  
GCTATATGTAAGCGGAAACCACCATTAATACTATCTGAACCTGTTGAACG

501 TGGTAATCTGAGAGAAAAATATCGAGTTGGGAAATGGTCCACTAGAGGAGG  
ACCATTAGACTCTCTTTTATAGCTCAACCCTTTACCAGGTGATCTCCTCC

551 CTATCTCAGCGCTTTATTATTACAGTACTGGTGGCACTCAGCTTCCAAC  
GATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA

601 CTGGCTCGTTCCCTTTATAATTGTCATCCAAATGATTTTCAAGCAGCAAG  
GACCGAGCAAGGAAATATTAAACGTAGGTTTACTAAAGTCTTCGTCGTTT

651 ATTCCAATATATTGAGGGAGAAATGCGCACGAGAAATTAGGTACAACCGGA  
TAAGGTTATATAACTCCCTCTTTACGCGTGCTCTTAATCCATGTTGGCCT

701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAAATAGTTGGGGGAGA  
CTAGACGTGGTCTAGGATCGCATTAATGTGAACCTTATCAACCCCTCT

751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTTGCTAGTCCAAT  
GAAAGGTGACGTTAAGTTCTCAGATTGGTTCCCTCGGAAACGATCAGGTTA

801 TCAACTGCAAAGACGTAATGGTTCCAAATTCAGTGTGTACGATGTGAGTA  
AGTTGACGTTTCTGCATTACCAAGGTTAAGTCACACATGCTACACTCAT

851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGCACCTCCACCA  
ATAATTAGGGATAGTATCGAGAGTACCACATATCTACGCGTGGAGGTGGT

901 TCGTCACAGTTTTCTGTGTATTTACAGGCATCGACGGGATATGGTAATGC  
AGCAGTGTCAAAGACACATAAATGTCCGTAGCTGCCCTATACCATTACG

**FIGURE 15D (CONT'D)**

951 TGATGTTTGTATGGATCCTGAGCCCATAGTGCGTATCGTAGGTCGAAATG  
ACTACAAACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTTAC

1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA  
CAGATACACAACATAATCCCTACCTTCTAAGGTGTTGCCTTTGCGTTAT

1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT  
GTCAACACCGGTACGTTTACAGATTATGTCACGTTTAGTCGAGACCTGAAA

1101 GAAAAGAGACAATACTATTTCGATCTAATGGAAAGTGTTTAACTACTTACG  
CTTTTCTCTGTTATGATAAGCTAGATTACCTTTCACAAATTGATGAATGC

1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA  
CCATGTACGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT

1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC  
TGACTACGGTGGGCGACCGTTTATACCCTATTACCTTGGTAGTATTTAGG

1251 CAGATCTAGTCTAGTTTTAGCAGCGACATCAGGGAACAGTGGTACCACAC  
GTCTAGATCAGATCAAAATCGTCGCTGTAGTCCCTTGTCACCATGGTGTG

1301 TTACAGTGCAAACCAACATTTATGCCGTTAGTCAAGGTTGGCTTCCTACT  
AATGTCACGTTTGGTTGTAAATACGGCAATCAGTTCCAACCGAAGGATGA

1351 AATAATACACAACCTTTTGTACAACCATTTGTTGGGCTATATGGTCTGTG  
TTATTATGTGTTGGAAAACAATGTTGGTAACAACCGATATACCAGACAC

1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA  
GAACGTTTCGTTTATCACCTGTTTATACCTATCTCCTGACATCGTCACTTT

1451 AGGCTGAACAACAGTGGGCTCTTTATGCAGATGGTTCAATACGTCCTCAG  
TCCGACTTGTTGTACACCGAGAAATACGTCTACCAAGTTATGCAGGAGTC

1501 CAAAACCGAGATAATTGCCTTACAAGTGATTCTAATATACGGGAAACAGT  
GTTTTGGCTCTATTAACGGAATGTTCACTAAGATTATATGCCCTTTGTCA

1551 TGTTAAGATCCTCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT  
ACAATTCTAGGAGAGAACACCGGGACGTAGGAGACCGGTTGCTACCTACA

1601 TCAAGAATGATGGAACCATTTTAAATTTGTATAGTGGATTGGTGTAGAT  
AGTTCCTTACTACCTTGGTAAATTTAAACATATCACCTAACCAATCTA

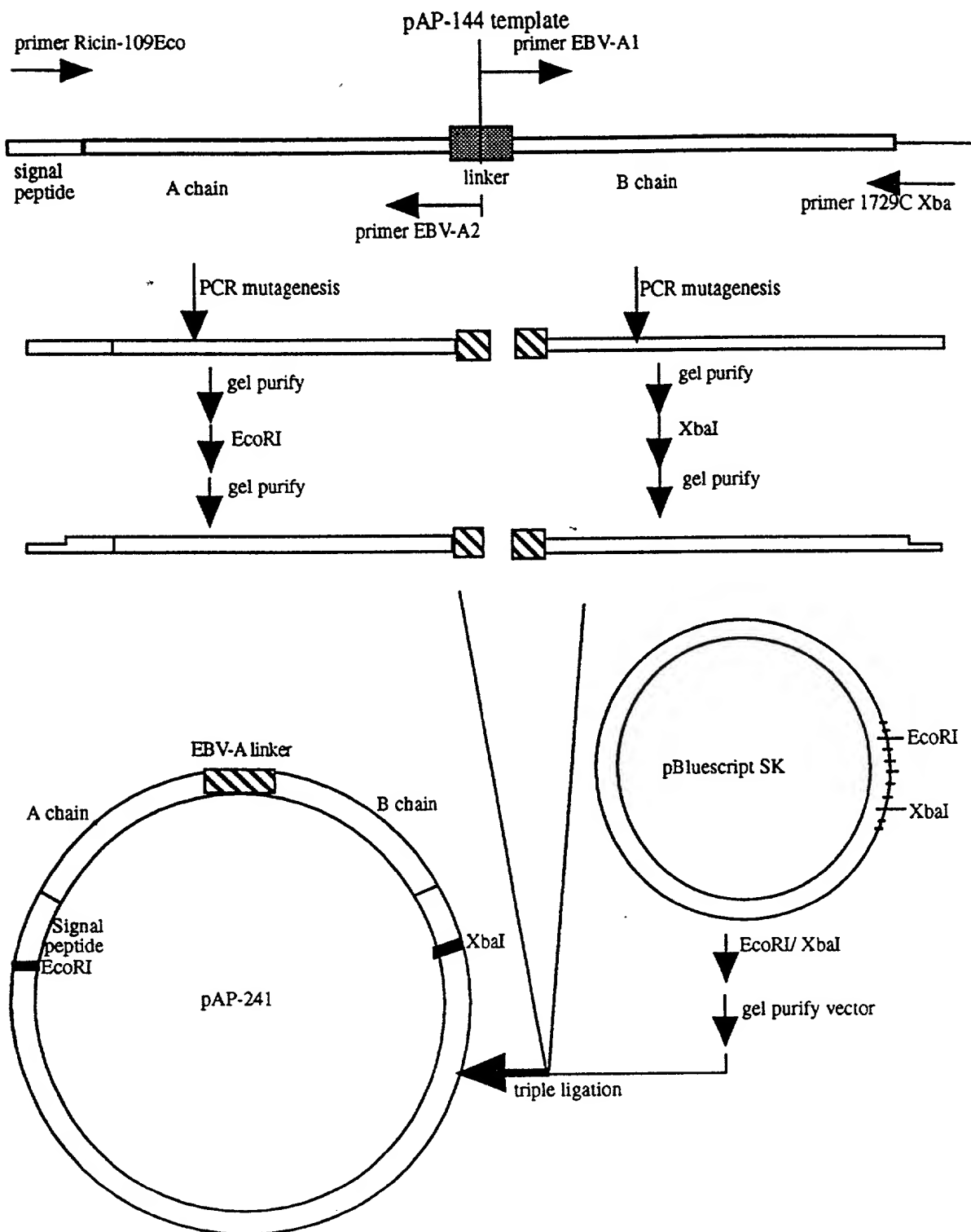
1651 GTGAGGCGATCGGATCCGAGCCTTAAACAAATCATTCTTTACCCTCTCCA  
CACTCCGCTAGCCTAGGCTCGGAATTTGTTTAGTAAGAAATGGGAGAGGT

1701 TGGTGACCCAAACCAAATATGGTTACCATTATTTTGATAGACAGATTACT  
ACCACTGGGTTTGGTTTATACCAATGGTAATAAACTATCTGTCTAATGA

1751 CTCTTGCAGTGTGTGTGTCCTGCCATGAAAATAGATGGCTTAAATAAAAA  
GAGAACGTCACACACACAGGACGGTACTTTTATCTACCGAATTTATTTTT

1801 GGACATTGTAAATTTTGTAACTGAAAGGACAGCAAGTTATATCGAATTCC  
CCTGTAAACATTTAAACATTGACTTTCCTGTCGTTCAATATAGCTTAAGG

1851 TGCAG  
ACGTC

**FIGURE 16A****PCR Mutagenesis of Preprorizin Gene to Create an EBV-A Variant Gene a) Cloning Strategy**

**FIGURE 16B**

**WT preprorin linker**

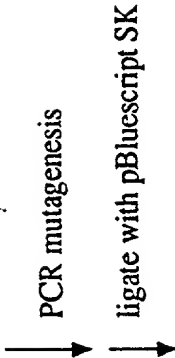
primer EBV-A1

5' - TCGGCGTCAGGTGTTAATGCTGATGTTTGT -3'

TCCTTGCTTATAAGGCCAGTGGTGCCAAATTTTAAT  
AGAAACGAATATTCGGTCACCCAGGTTAAATAATTA

3' - AGCAGTGTCAAAAGATTTCGAACATGTCCGT -5'

primer EBV-A2

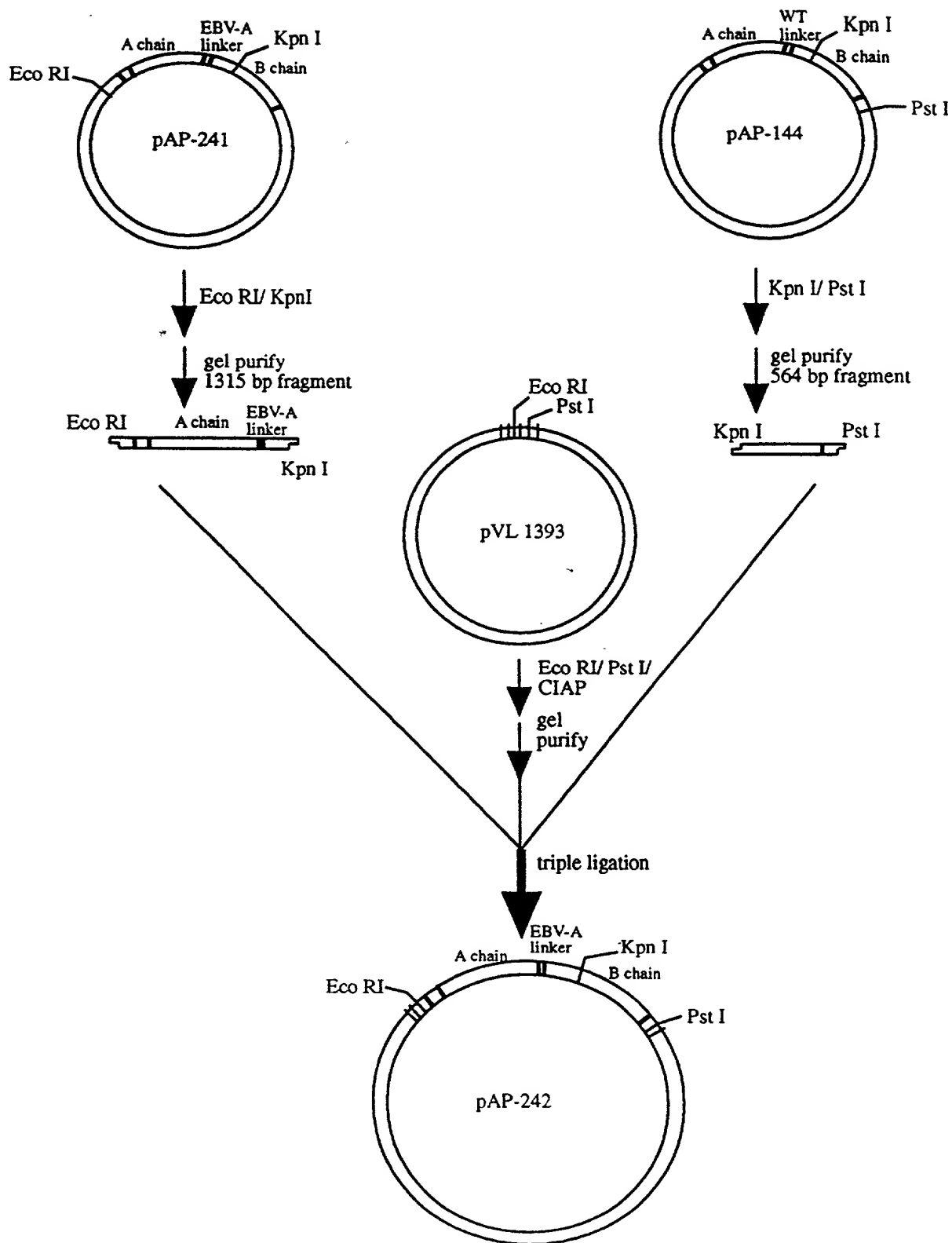


**pAP 241 linker  
(EBV-A variant)**

TCTAAGCTTGACAGGCATCGGCGTCAGGTGTTAAT  
AGATTGGAACATGTCCGTAGCCGCGAGTCCACAATTA

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**FIGURE 16C**



00440" 151550



**FIGURE 16D**

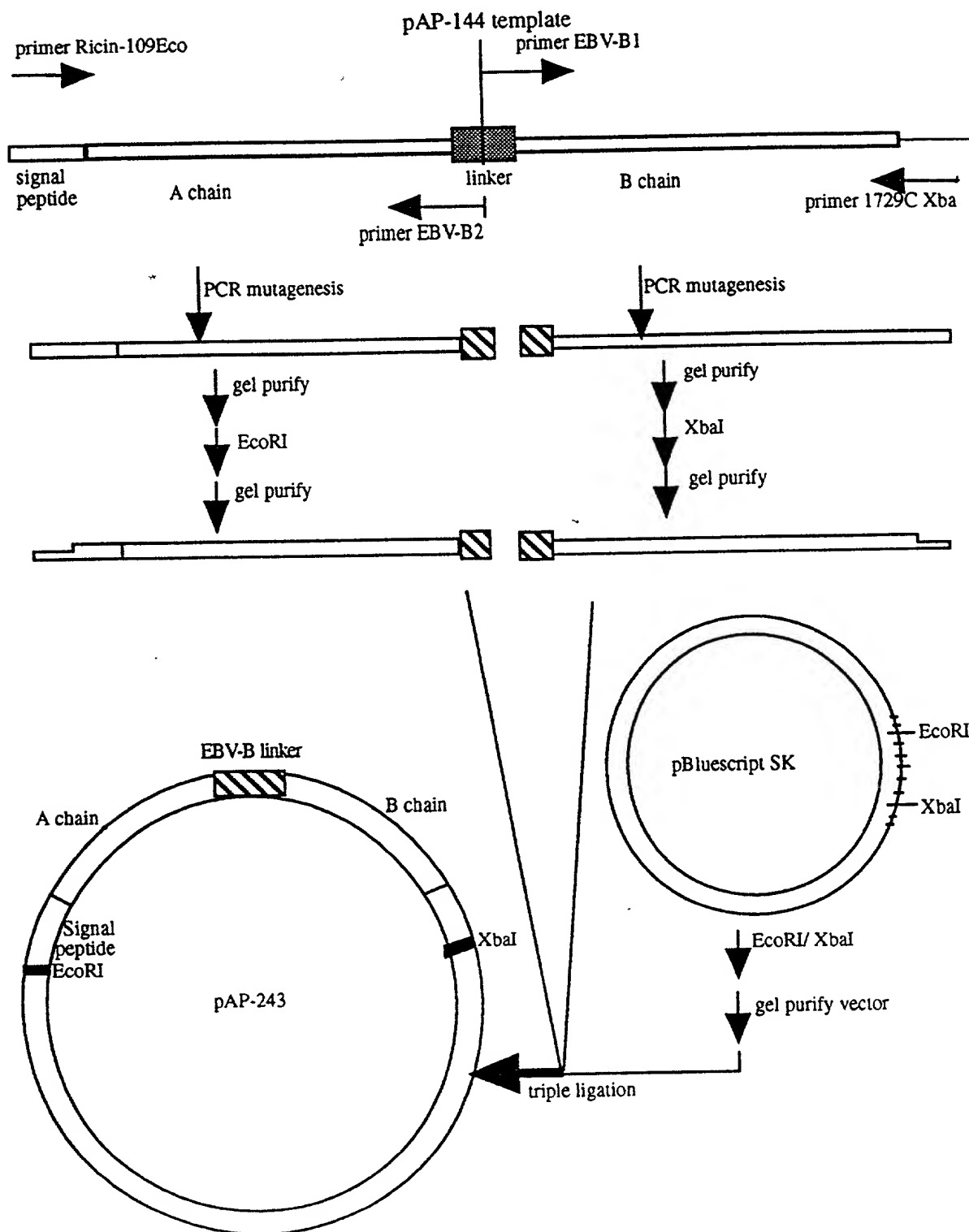
10 20 30 40 50  
 1 GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT  
 CTTAAGTACTTTGGCCCTCCTTTATGATAACATTATACCTACATACGTCA  
 51 GGCAACATGGCTTTGTTTTGGATCCACCTCAGGGTGGTCTTTCACATTAG  
 CCGTTGTACCGAAACAAACCTAGGTGGAGTCCCACCAGAAAGTGAATC  
 101 AGGATAACAACATATTCCCCAAACAATACCCAATTATAAACTTTACCACA  
 TCCTATTGTTGTATAAGGGGTTTGTATGGGTAAATATTTGAAATGGTGT  
 151 GCGGGTGCCACTGTGCAAAGCTACACAACTTTATCAGAGCTGTTGCGGG  
 CGCCACGGTGACACGTTTCGATGTGTTTGAAATAGTCTCGACAAGCGCC  
 201 TCGTTTAACAACCTGGAGCTGATGTGAGACATGATATACCAGTGTGCCAA  
 AGCAAATGTTGACCTCGACTACACTCTGTACTATATGGTCACAACGGTT  
 251 ACAGAGTTGGTTTGCCTATAAAACCAACGGTTTATTTTAGTTGAACTCTCA  
 TGTCTCAACCAACGGATATTTGGTTGCCAAATAAAATCAACTTGAGAGT  
 301 AATCATGCAGAGCTTTCTGTGTACATTAGCGCTGGATGTCACCAATGCATA  
 TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT  
 351 TGTGGTCGGCTACCGTGCTGGAAATAGCGCATATTTCTTTTCATCCTGACA  
 ACACCAGCCGATGGCAGGACCTTTATCGCGTATAAAGAAAGTAGGACTGT  
 401 ATCAGGAAGATGCAGAAACCAATCACTCATCTTTTCACTGATGTTCAAAAT  
 TAGTCCTTCTACGTCTTCGTTAGTGAGTAGAAAAGTGAAGTACAAGTTTGA  
 451 CGATATACATTTCGCCTTTGGTGGTAATTATGATAGACTTGAACAACCTTGC  
 GCTATATGTAAGCGGAAACCACCAATTAATACTATCTGAACCTGTTGAACG  
 501 TGGTAATCTGAGAGAAAATATCGAGTTGGGAAATGGTCCACTAGAGGAGG  
 ACCATTAGACTCTCTTTTATAGCTCAACCCTTTACCAGGTGATCTCCTCC  
 551 CTATCTCAGCGCTTTATTATTACAGTACTGGTGGCACTCAGCTTCCAACCT  
 GATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA  
 601 CTGGCTCGTTCTTTTATAATTTGCATCCAAATGATTTTCAAGCAGCAAG  
 GACCGAGCAAGGAAATATTAAACGTAGGTTTACTAAAGTCTTCGTCGTTC  
 651 ATTCCAATATATTGAGGGAGAAATGCGCAGGAGAAATAGGTACAACCGGA  
 TAAGGTTATATAACTCCCTCTTTACGCGTGCTCTTAATCCATGTTGGCCT  
 701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGGAGA  
 CTAGACGTGGTCTAGGATCGCATTAATGTGAACCTCTTATCAACCCCTCT  
 751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTTGCTAGTCCAAT  
 GAAAGGTGACGTTAAGTTCTCAGATTGGTTCTCGGAAACGATCAGGTTA  
 801 TCAACTGCAAAGACGTAATGGTTCCAAATTCAGTGTGTACGATGTGAGTA  
 AGTTGACGTTTCTGCATTACCAAGGTTTAAGTCACACATGCTACACTCAT  
 851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGCACCTCCACCA  
 ATAATTAGGGATAGTATCGAGAGTACCACATATCTACGCGTGAGGTGGT  
 901 TCGTCACAGTTTTCTAAGCTTGTACAGGCATCGGCGTCAGGTGTTAATGC  
 AGCAGTGTCAAAAGATTGGAACATGTCCGTAGCCGCAGTCCACAATTACG

**FIGURE 16D (CONT'D)**

951 TGATGTTTGTATGGATCCTGAGCCCATAGTGCATCGTAGGTCGAAATG  
 ACTACAAACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTTAC  
 1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAACGCAATA  
 CAGATACACAACCTACAATCCCTACCTTCTAAGGTGTTGCCTTTGCGTTAT  
 1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT  
 GTCAACACCGGTACGTTTCAAGTTATGTCTACGTTTAGTCGAGACCTGAAA  
 1101 GAAAAGAGACAATACTATTTCGATCTAATGGAAAGTGTAACTACTTACG  
 CTTTTCTCTGTTATGATAAGCTAGATTACCTTTTACAAATTGATGAATGC  
 1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA  
 CCATGTCAGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT  
 1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC  
 TGAATACGTTGGGCGACCGTTTATACCTATTACCTTGGTAGTATTTAGG  
 1251 CAGATCTAGTCTAGTTTATGACGCGACATCAGGGAACAGTGGTACCACAC  
 GTCTAGATCAGATCAAAATCGTCGCTGTAGTCCCTTGTCAACCATGGTGTG  
 1301 TTACAGTGCAAACCAACATTTATGCCGTTAGTCAAGGTTGGCTTCCTACT  
 AATGTCACGTTTGGTTGTAAATACGGCAATCAGTTCCAACCGAAGGATGA  
 1351 AATAATACACAACCTTTTGTGTTACAACCATTTGTTGGGCTATATGGTCTGTG  
 TTATTATGTGTTGGAAAACATGTTGGTAACAACCCGATATACCAGACAC  
 1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA  
 GAACGTTTCGTTTATCACCTGTTTCATACCTATCTCCTGACATCGTCACTTT  
 1451 AGGCTGAACAACAGTGGGCTCTTTATGCAGATGGTTCAATACGTCCTCAG  
 TCCGACTTGTGTTGTCACCCGAGAAATACGTCCTACCAAGTTATGCAGGAGTC  
 1501 CAAAACCGAGATAATTGCCTTACAAGTGATTCTAATATACGGGAAACAGT  
 GTTTTGGCTCTATTAAACGGAATGTTCACTAAGATTATATGCCCTTTGTCA  
 1551 TGTTAAGATCCTCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT  
 ACAATTCTAGGAGAGAACACCGGGACGTAGGAGACCGGTTGCTACCTACA  
 1601 TCAAGAATGATGGAACCATTTTAAATTTGTATAGTGGATTGGTGTTAGAT  
 AGTTCTTACTACCTTGGTAAATTTAAACATATCACCTAACCAATCTA  
 1651 GTGAGGCGATCGGATCCGAGCCTTAAACAAATCATTCTTTACCCCTCTCCA  
 CACTCCGCTAGCCTAGGCTCGGAATTTGTTTAGTAAGAAATGGGAGAGGT  
 1701 TGGTGACCCAAACCAAATATGGTTACCATTATTTTGATAGACAGATTACT  
 ACCACTGGGTTTGGTTTATACCAATGGTAATAAACTATCTGTCTAATGA  
 1751 CTCTTGCAGTGTGTGTGCTGCGCATGAAAATAGATGGCTTAAATAAAAA  
 GAGAACGTCACACACACAGGACGGTACTTTTATCTACCGAATTTATTTTT  
 1801 GGACATTGTAAATTTTGTAACTGAAAGGACAGCAAGTTATATCGAATTCC  
 CCTGTAAACATTTAAACATTGACTTTCCTGTCGTTCAATATAGCTTAAGG  
 1851 TGCAG  
 ACGTC

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**FIGURE 17A**



## FIGURE 17B

### WT preprorin linker

primer EBV-B1

5' - TCGGACGCACCTGATAATGCTGATGTTTGT - 3'

TCTTTGCTTATAAGGCCAGTGGTGCCAAATTTTAAT  
AGAAACGAATATTCGGTCACCCACGGTTAAAATTA

3' - AGCAGTGTCAAAAGAACATAGATTTCGGT - 5'

primer EBV-B2

PCR mutagenesis

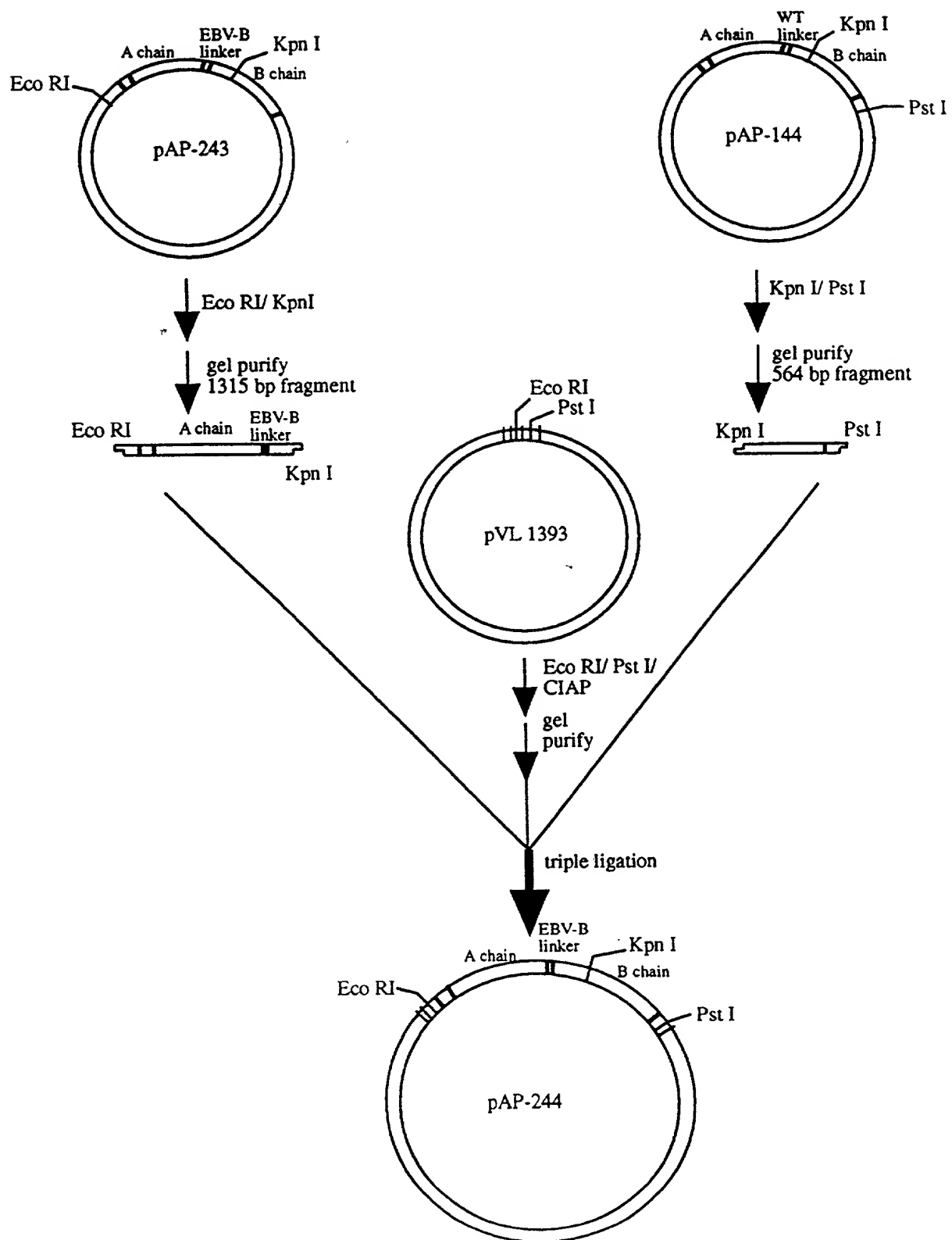
ligate with pBluescript SK

pAP 243 linker  
(EBV-B variant)

TCTTCGTATCTAAAGGCATCGGACGCACCTGATAAT  
AGAACATAGATTTCGGTAGCCTGCCGTGGACTATTA

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**FIGURE 17C**



004740 "TST560

85/254

# FIGURE 17D

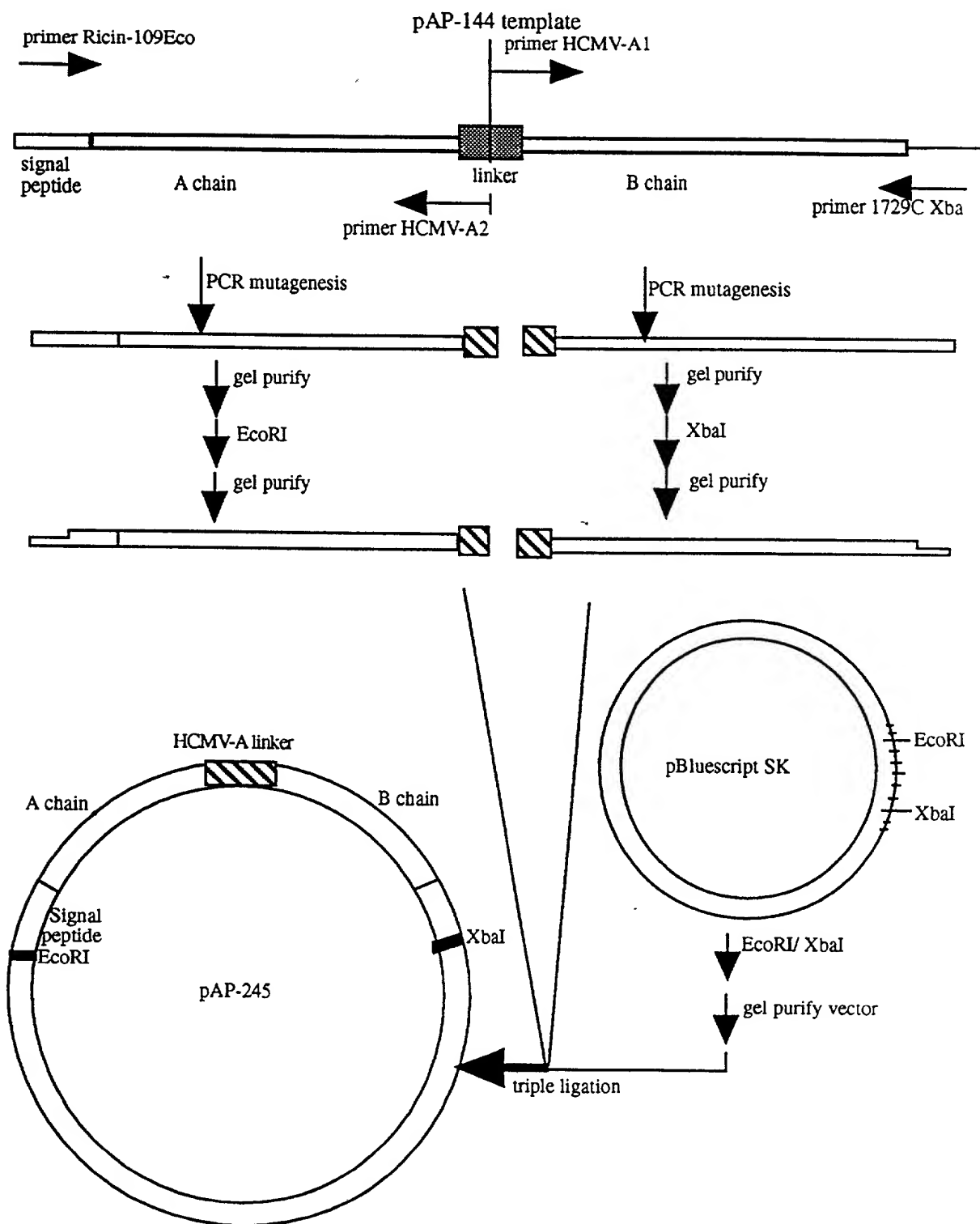
	10	20	30	40	50
1	GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT				
	CTTAAGTACTTTGGCCCTCCTTTATGATAACATTATACCTACATACGTCA				
51	GGCAACATGGCTTTGTTTTGGATCCACCTCAGGGTGGTCTTTCACATTAG				
	CCGTTGTACCGAAACAAACCTAGGTGGAGTCCCACCAGAAAGTGTAATC				
101	AGGATAACAACATATTCCCCAAACAATACCCAATTATAAACTTTACCACA				
	TCCTATTGTTGTATAAGGGGTTTGTTATGGGTTAATATTTGAAATGGTGT				
151	GCGGGTGCCACTGTGCAAAGCTACACAACTTTATCAGAGCTGTTTCGCGG				
	CGCCACGGTGACACGTTTCGATGTGTTTGAAATAGTCTCGACAAGCGCC				
201	TCGTTTAACTGAGCTGATGTGAGACATGATATACCAGTGTTCGCAA				
	AGCAAATTGTTGACCTCGACTACACTCTGTACTATATGGTCACAACGGTT				
251	ACAGAGTTGGTTTGCCCTATAAACCACGGTTTATTTTAGTTGAACCTCA				
	TGTCTCAACCAAACGGATATTTGGTTGCCAAATAAATCAACTTGAGAGT				
301	AATCATGCAGAGCTTTCTGTTACATTAGCGCTGGATGTCACCAATGCATA				
	TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT				
351	TGTGGTCGGCTACCGTGCTGGAAATAGCGCATATTTCTTTTCATCCTGACA				
	ACACCAGCCGATGGCACGACCTTTATCGCGTATAAAGAAAGTAGGACTGT				
401	ATCAGGAAGATGCAGAAGCAATCACTCATCTTTTCACTGATGTTCAAAT				
	TAGTCCTTCTACGTCTTCGTTAGTGAGTAGAAAAGTGAAGTACAAGTTTTA				
451	CGATATACATTTCGCTTTGGTGGTAATTATGATAGACTTGAACAACCTGC				
	GCTATATGTAAGCGGAAACCACCATTAACTATCTGAAGTGTGTAACG				
501	TGGTAATCTGAGAGAAAATATCGAGTTGGGAAATGGTCCACTAGAGGAGG				
	ACCATTAGACTCTCTTTTATAGCTCAACCCTTTACCAGGTGATCTCCTCC				
551	CTATCTCAGCGCTTTATTATTACAGTACTGGTGGCACTCAGCTTCCAAC				
	GATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA				
601	CTGGCTCGTTCCTTTATAATTTGCATCCAAATGATTTCAGAAGCAGCAAG				
	GACCGAGCAAGGAAATATTAAACGTAGGTTTACTAAAGTCTTCGTCGTTT				
651	ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA				
	TAAGGTTATATAACTCCCTCTTTACGCGTGCTCTTAATCCATGTTGGCCT				
701	GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGGAGA				
	CTAGACGTGGTCTAGGATCGCATTAAATGTGAAGTCTTATCAACCCCTCT				
751	CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTTGCTAGTCCAAT				
	GAAAGGTGACGTTAAGTTCTCAGATTGGTTCTCGGAAACGATCAGGTTA				
801	TCAACTGCAAAGACGTAATGGTTCCAAATTCAGTGTGTACGATGTGAGTA				
	AGTTGACGTTTCTGCATTACCAAGGTTTAAGTCACACATGCTACACTCAT				
851	TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGCACCTCCACCA				
	ATAATTAGGGATAGTATCGAGAGTACCACATATCTACGCGTGAGGTGGT				
901	TCGTCACAGTTTCTTCGTATCTAAAGGCATCGGACGCACCTGATAATGC				
	AGCAGTGTCAAAGAAGCATAGATTTCGCTAGCCTGCGTGGAATATTACG				

**FIGURE 17D (CONT'D)**

951 TGATGTTTGTATGGATCCTGAGCCCATAGTGCGTATCGTAGGTCGAAATG  
 ACTACAAACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTTAC  
 1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA  
 CAGATACACAACACTACAATCCCTACCTTCTAAGGTGTTGCCTTTGCGTTAT  
 1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT  
 GTCAACACCGGTACGTTTACGATTATGTCTACGTTTAGTCGAGACCTGAAA  
 1101 GAAAAGAGACAATACTATTTCGATCTAATGGAAAGTGTTTAACTACTTACG  
 CTTTTCTCTGTTATGATAAGCTAGATTACCTTTCACAAATTGATGAATGC  
 1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA  
 CCATGTCAGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT  
 1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC  
 TGACTACGGTGGGCGACCGTTTATACCCTATTACCTTGGTAGTATTTAGG  
 1251 CAGATCTAGTCTAGTTTTAGCAGCGACATCAGGGAACAGTGGTACCACAC  
 GTCTAGATCAGATCAAAATCGTCGCTGTAGTCCCTTGTCAACCATGGTGTG  
 1301 TTACAGTGCAAACCAACATTTATGCCGTTAGTCAAGGTTGGCTTCCTACT  
 AATGTCACGTTTGGTTGTAAATACGGCAATCAGTTCCAACCGAAGGATGA  
 1351 AATAATACACAACCTTTTGTGTTACAACCATTTGTTGGGCTATATGGTCTGTG  
 TTATTATGTGTTGGAAAAACAATGTTGGTAACAACCCGATATACCAGACAC  
 1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA  
 GAACGTTTCGTTTATCACCTGTTTCATACCTATCTCCTGACATCGTCACTTT  
 1451 AGGCTGAACAACAGTGGGCTCTTTATGCAGATGGTTCAATACGTCCTCAG  
 TCCGACTTGTGTCACCCGAGAAATACGTCTACCAAGTTATGCAGGAGTC  
 1501 CAAAACCGAGATAATTGCCTTACAAGTGATTCTAATATACGGGAAACAGT  
 GTTTTGGCTCTATTAAACGGAATGTTCACTAAGATTATATGCCCTTTGTCA  
 1551 TGTTAAGATCCTCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT  
 ACAATTC TAGGAGAGAACACCGGGACGTAGGAGACCGGTTGCTACCTACA  
 1601 TCAAGAATGATGGAACCATTTTAAATTTGTATAGTGGATTGGTGTTAGAT  
 AGTTCCTTACTACCTTGGTAAAAATTTAAACATATCACCTAACCAATCTA  
 1651 GTGAGGCGATCGGATCCGAGCCTTAAACAAATCATTCTTTACCCCTCTCCA  
 CACTCCGCTAGCCTAGGCTCGGAATTTGTTTAGTAAGAAATGGGAGAGGT  
 1701 TGGTGACCCAAACCAAATATGGTTACCATTATTTTGATAGACAGATTACT  
 ACCACTGGGTTTGGTTTATACCAATGGTAATAAACTATCTGTCTAATGA  
 1751 CTCCTGCAGTGTGTGTGTCTCGCCATGAAAATAGATGGCTTAAATAAAAA  
 GAGAACGTCACACACACAGGACGGTACTTTTATCTACCGAATTTATTTTT  
 1801 GGACATTGTAAATTTTGTAACTGAAAGGACAGCAAGTTATATCGAATTCC  
 CCTGTAACATTTAAACATTGACTTTCCTGTCGTTCAATATAGCTTAAGG  
 1851 TGCAG  
 ACGTC

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**FIGURE 18A**





**FIGURE 18B**

WT preprorin linker

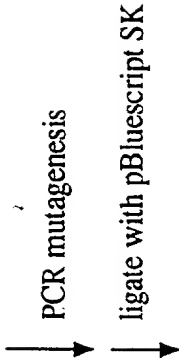
primer HCMV-A1

5' - TCGGTAGACTTGCTAATGCTGATGTTGT -3'

TCTTTGCTTATAAGGCCACTGGTGCCAAATTTTAAT  
AGAAACGAATATTCCGGTCACCCACGGTTAAATTA

3' - AGCAGTGTCAAAAGACCCCAACATTTACGT-5'

primer HCMV-A2

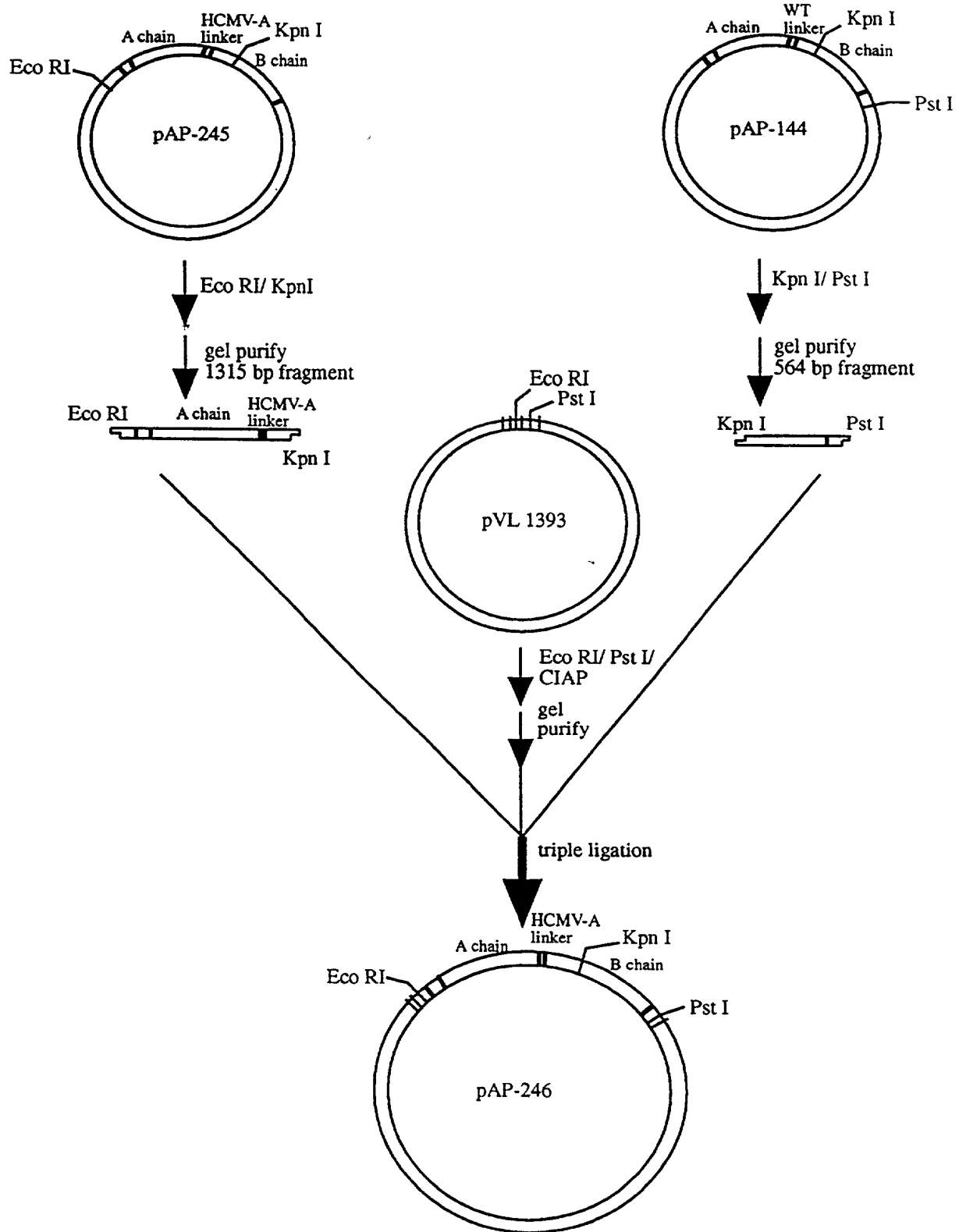


pAP 245 linker  
(HCMV-A variant)

TCTGGGGTTGTAATGCATCGGTAGACTTGCTAAT  
AGACCCCAACATTTACGTAGCACATCTGAACGATTA

89/254

**FIGURE 18C**



004740"TSF550

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## FIGURE 18D

10 20 30 40 50  
1 GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT  
CTTAAGTACTTTGGCCCTCCTTTATGATAACATTATACCTACATACGTCA  
51 GGCAACATGGCTTTGTTTTGGATCCACCTCAGGGTGGTCTTTCACATTAG  
CCGTTGTACCGAAACAAAACCTAGGTGGAGTCCCACCAGAAAGTGAATC  
101 AGGATAACAACATATTCCCCAAACAATACCCAATTATAAACTTTACCACA  
TCCTATTGTTGTATAAGGGGTTTGTATGGGTAAATATTTGAAATGGTGT  
151 GCGGGTGCCACTGTGCAAAGCTACACAACTTTATCAGAGCTGTTGCGGG  
CGCCACGGTGACACGTTTCGATGTGTTTGAAATAGTCTCGACAAGCGCC  
201 TCGTTTAAACAACTGGAGCTGATGTGAGACATGATATACCAGTGTGCCAA  
AGCAAATTGTTGACCTCGACTACACTCTGTACTATATGGTCACAACGGTT  
251 ACAGAGTTGGTTTGCCTATAAACCAACGGTTTATTTTAGTTGAACTCTCA  
TGTCTCAACCAACGGATATTTGGTTGCCAAATAAAATCAACTGAGAGT  
301 AATCATGCAGAGCTTTCTGTTACATTAGCGCTGGATGTCACCAATGCATA  
TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT  
351 TGTGGTCGGCTACCGTGCTGGAAATAGCGCATATTTCTTTCATCCTGACA  
ACACCAGCCGATGGCAGCAGCTTTATCGCGTATAAAGAAAGTAGGACTGT  
401 ATCAGGAAGATGCAGAAGCAATCACTCATCTTTTCACTGATGTTCAAAAT  
TAGTCCTTCTACGTCTTCGTTAGTGAGTAGAAAAGTGACTACAAGTTTTA  
451 CGATATACATTTCGCCCTTTGGTGGTAATTATGATAGACTTGAACAACTTGC  
GCTATATGTAAGCGGAAACCACCATTAATACTATCTGAACCTGTTGAACG  
501 TGGTAATCTGAGAGAAAAATATCGAGTTGGGAAATGGTCCACTAGAGGAGG  
ACCATTAGACTCTCTTTTATAGCTCAACCCTTTACCAGGTGATCTCCTCC  
551 CTATCTCAGCGCTTTATTATTACAGTACTGGTGGCACTCAGCTTCCAAC  
GATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA  
601 CTGGCTCGTTCCCTTTATAATTTGCATCCAAATGATTTTCAAGCAGCAAG  
GACCGAGCAAGGAAATATTAAACGTAGGTTTACTAAAGTCTTCGTCGTTT  
651 ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA  
TAAGGTTATATACTCCCTCTTTACGCGTGCTCTTAATCCATGTTGGCCT  
701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGGAGA  
CTAGACGTGGTCTAGGATCGCATTAATGTGAACCTTATCAACCCCTCT  
751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTTGCTAGTCCAAT  
GAAAGGTGACGTTAAGTTCTCAGATTGGTTCCTCGGAAACGATCAGGTTA  
801 TCAACTGCAAAGACGTAATGGTTCCAAATTCAGTGTGTACGATGTGAGTA  
AGTTGACGTTTCTGCATTACCAAGGTTTAAGTCACACATGCTACACTCAT  
851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGCACCTCCACCA  
ATAATTAGGGATAGTATCGAGAGTACCACATATCTACGCGTGGAGGTGGT  
901 TCGTCACAGTTTTCTGGGGTTGTAAATGCATCGTGTAGACTTGCTAATGC  
AGCAGTGTCAAAGACCCCAACATTTACGTAGCACATCTGAACGATTACG

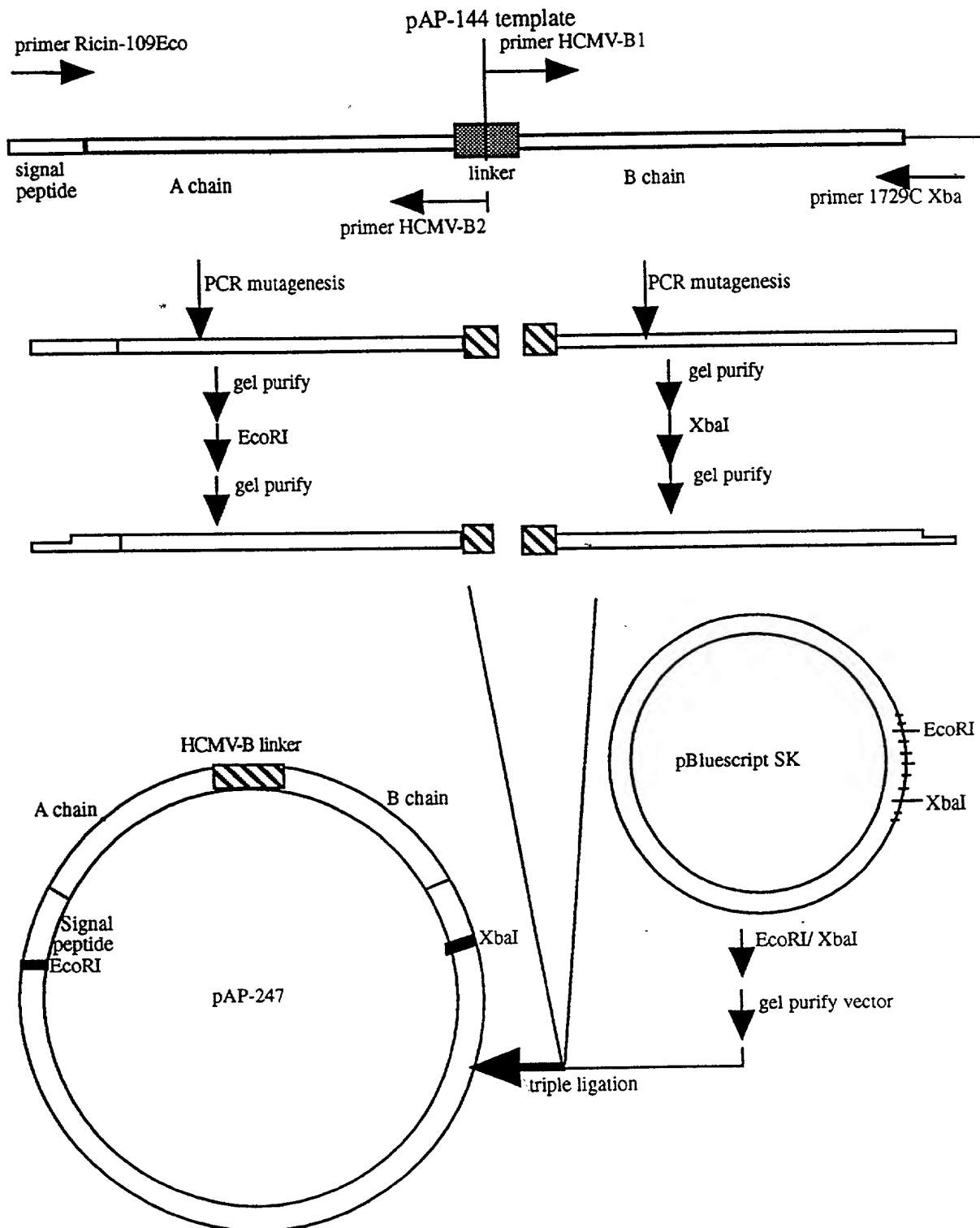
095451 "041400

**FIGURE 18D (CONT'D)**

951 TGATGTTTGTATGGATCCTGAGCCCATAGTGCATCGTAGGTCGAAATG  
 ACTACAAACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTTAC  
 1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA  
 CAGATACACAACCTACAATCCCTACCTTCTAAGGTGTTGCCTTTGCGTTAT  
 1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT  
 GTCAACACCGGTACGTTTACGATTATGTCTACGTTTAGTCGAGACCTGAAA  
 1101 GAAAAGAGACAATACTATTCGATCTAATGGAAAGTGTTTAACTACTTACG  
 CTTTTCTCTGTTATGATAAGCTAGATTACCTTTCACAAATTGATGAATGC  
 1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA  
 CCATGTCAGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT  
 1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC  
 TGACTACGGTGGGCGACCGTTTATACCCTATTACCTTGGTAGTATTTAGG  
 1251 CAGATCTAGTCTAGTTTTAGCAGCGACATCAGGGAACAGTGGTACCACAC  
 GTCTAGATCAGATCAAAATCGTCGCTGTAGTCCCTTGTCACCATGGTGTG  
 1301 TTACAGTGCAAACCAACATTTATGCCGTTAGTCAAGGTTGGCTTCCTACT  
 AATGTCACGTTTGGTTGTAAATACGGCAATCAGTTCCAACCGAAGGATGA  
 1351 AATAATACACAACCTTTTGTACAACCATTGTTGGGCTATATGGTCTGTG  
 TTATTATGTGTTGGAAAACAATGTTGGTAACAACCCGATATACCAGACAC  
 1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA  
 GAACGTTTCGTTTATCACCTGTTTACATACCTATCTCCTGACATCGTCACTTT  
 1451 AGGCTGAACAACAGTGGGCTCTTTATGCAGATGGTTCAATACGTCCTCAG  
 TCCGACTTGTGTGTCACCCGAGAAATACGTCTACCAAGTTATGCAGGAGTC  
 1501 CAAAACCGAGATAATTGCCTTACAAGTGATTCTAATATACGGGAAACAGT  
 GTTTTGGCTCTATTAACGGAATGTTCACTAAGATTATATGCCCTTTGTCA  
 1551 TGTTAAGATCCTCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT  
 ACAATTCTAGGAGAGAACACCGGGACGTAGGAGACCGGTTGCTACCTACA  
 1601 TCAAGAATGATGGAACCATTTTAAATTTGTATAGTGGATTGGTGTAGAT  
 AGTTCTTACTACCTTGGTAAAATTTAAACATATCACCTAACCAATCTA  
 1651 GTGAGGCGATCGGATCCGAGCCTTAAACAAATCATTCTTTACCCTCTCCA  
 CACTCCGCTAGCCTAGGCTCGGAATTTGTTTAGTAAGAAATGGGAGAGGT  
 1701 TGGTGACCCAAACCAATATGGTTACCATTATTTTGATAGACAGATTACT  
 ACCACTGGGTTTGGTTTATACCAATGGTAATAAACTATCTGTCTAATGA  
 1751 CTCTTGCAAGTGTGTGTGTCCTGCCATGAAAATAGATGGCTTAAATAAAAA  
 GAGAACGTCACACACACAGGACGGTACTTTTATCTACCGAATTTATTTTT  
 1801 GGACATTGTAAATTTTGTAACTGAAAGGACAGCAAGTTATATCGAATTCC  
 CCTGTAACATTTAAACATTGACTTTCCTGTCGTTCAATATAGCTTAAGG  
 1851 TGCAG  
 ACGTC

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**FIGURE 19A**



## FIGURE 19B

### WT preproricin linker

primer HCMV-B1

5' - TCGGTGTCACCTGAAAAATGCTGATGTTTGT -3'

TCTTTGCTTATAAGGCCAGTGGTGCCAAATTTTAAT  
AGAAACGAATATTCGGTACCCACGGTTTAAATTA

3' - AGCAGTGTCAAAGAAGCATACATTTCCGT-5'

primer HCMV-B2

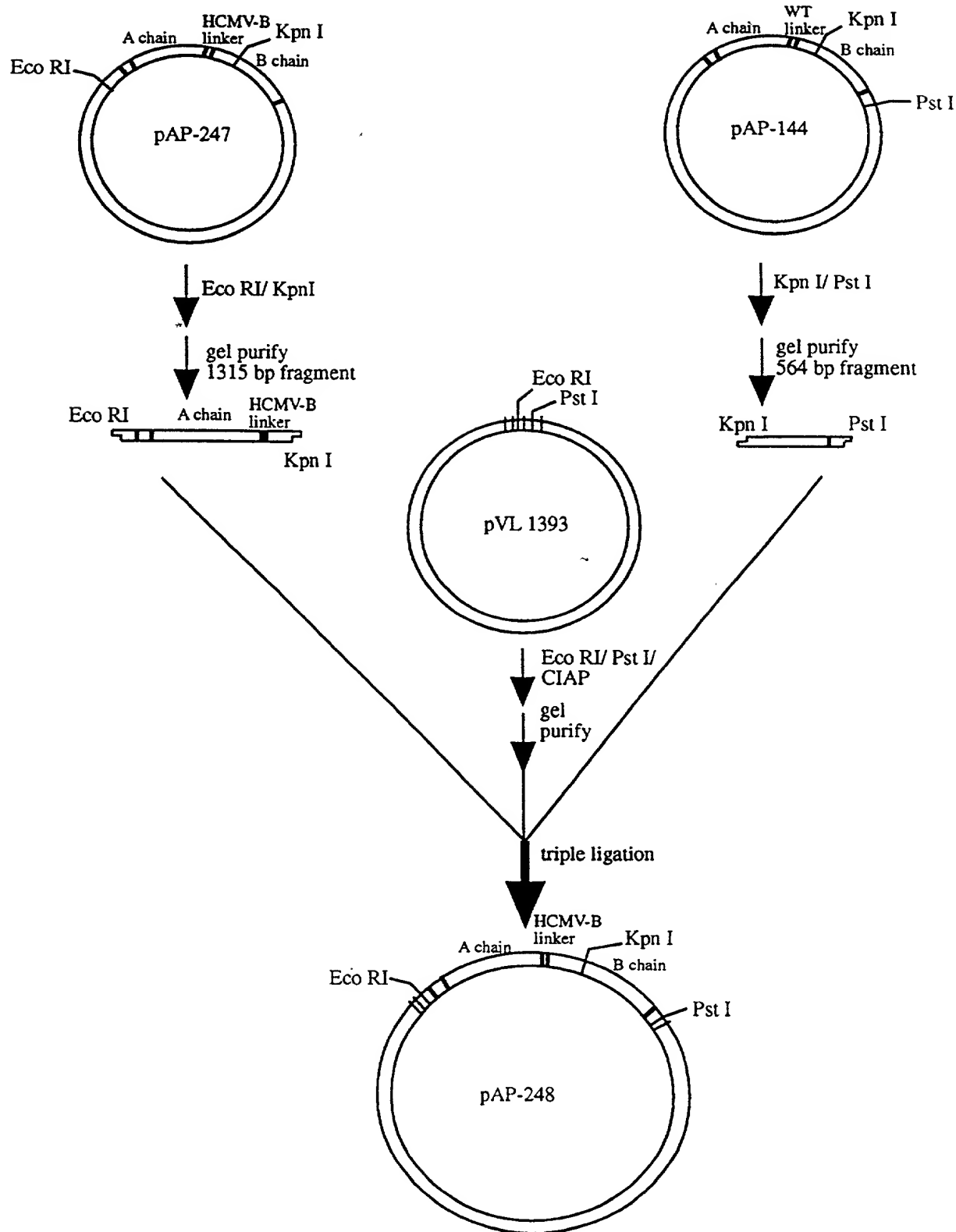
PCR mutagenesis  
ligate with pBluescript SK

pAP 247 linker  
(HCMV-B variant)

TCTTCGTATGTAAAGGCATCGGTGTCACCTGAAAT  
AGAACATACATTTCCGTAGCCACAGTGGACTTTA

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**FIGURE 19C**



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## FIGURE 19D

10 20 30 40 50  
1 GAATTCATGAAACCGGAGGAAATACTATTGTAATATGGATGTATGCAGT  
CTTAAGTACTTTGGCCCTCCTTTATGATAACATTATACCTACATACGTCA  
51 GGCAACATGGCTTTGTTTTGGATCCACCTCAGGGTGGTCTTTCACATTAG  
CCGTTGTACCGAAACAAAACCTAGGTGGAGTCCCACCAGAAAAGTGTAATC  
101 AGGATAACAACATATTTCCCAAACAATACCCAATTATAAACTTTACCACA  
TCCTATTGTTGTATAAGGGGTTTGTTATGGGTAAATATTTGAAATGGTGT  
151 GCGGGTGCCACTGTGCAAAGCTACACAACTTTATCAGAGCTGTTGCGGG  
CGCCACGGTGACACGTTTCGATGTGTTTGAAATAGTCTCGACAAGCGCC  
201 TCGTTTAACTGGAGCTGATGTGAGACATGATATACCAGTGTGCCAA  
AGCAAATTGTTGACCTCGACTACACTCTGTACTATATGGTCACAACGGTT  
251 ACAGAGTTGGTTTGCCCTATAAACCAACGGTTTATTTTAGTTGAACTCTCA  
TGTCTCAACCAAACGGATATTGGTTGCCAAATAAAATCAACTTGAGAGT  
301 AATCATGCAGAGCTTCTGTTACATTAGCGCTGGATGTCACCAATGCATA  
TTAGTACGTCTCGAAAGACAAATGTAATCGCGACCTACAGTGGTTACGTAT  
351 TGTGGTCGGCTACCGTGCTGGAAATAGCGCATATTTCTTTCATCCTGACA  
ACACCAGCCGATGGCAGACCTTTATCGCGTATAAAGAAAGTAGGACTGT  
401 ATCAGGAAGATGCAGAAGCAATCACTCATCTTTTCACTGATGTTCAAAT  
TAGTCCTTCTACGTCTTCGTTAGTGAGTAGAAAAGTGAAGTACAAGTTTCA  
451 CGATATACATTGCGCTTTGGTGGTAATTATGATAGACTTGAACAACCTGC  
GCTATATGTAAGCGGAAACCACCATTAATACTATCTGAACCTGTTGAACG  
501 TGGTAATCTGAGAGAAAATATCGAGTTGGGAAATGGTCCACTAGAGGAGG  
ACCATTAGACTCTCTTTTATAGCTCAACCTTTACCAGGTGATCTCCTCC  
551 CTATCTCAGCGCTTTATTATTACAGTACTGGTGGCACTCAGCTTCCAAC  
GATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA  
601 CTGGCTCGTTCCTTTATAATTTGCATCCAAATGATTTGAGAAGCAGCAAG  
GACCGAGCAAGGAAATATTAAACGTAGGTTTACTAAAGTCTTCGTCGTTT  
651 ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA  
TAAGGTTATATAACTCCCTCTTTACGCGTGCTCTTAATCCATGTTGGCCT  
701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGGAGA  
CTAGACGTGGTCTAGGATCGCATTAAATGTGAACCTTATCAACCCCTCT  
751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTTGCTAGTCCAAT  
GAAAGGTGACGTTAAGTTCTCAGATTGGTTCTCGGAAACGATCAGGTTA  
801 TCAACTGCAAAGACGTAATGGTTCCAAATTCAGTGTGTACGATGTGAGTA  
AGTTGACGTTTCTGCATTACCAAGGTTAAGTCACACATGCTACACTCAT  
851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGCACCTCCACCA  
ATAATTAGGGATAGTATCGAGAGTACCACATATCTACGCGTGGAGGTGGT  
901 TCGTCACAGTTTTCTTCGTATGTAAAGGCATCGGTGTCACCTGAAAATGC  
AGCAGTGTCAAAAGAAGCATAACATTTCCGTAGCCACAGTGGACTTTTACG

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**FIGURE 19D (CONT'D)**

951 TGATGTTTGTATGGATCCTGAGCCCATAGTGCCTATCGTAGGTCGAAATG  
ACTACAAACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTTAC

1001 GTCCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA  
CAGATACACAAC TACAATCCCTACCTTCTAAGGTGTTGCCTTTGCGTTAT

1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT  
GTCAACACCGGTACGTTTCAAGATTATGTCTACGTTTGTGCGAGACCTGAAA

1101 GAAAAGAGACAATACTATTTCGATCTAATGGAAAGTGTTTAACTACTTACG  
CTTTTCTCTGTTATGATAAGCTAGATTACCTTTCACAAATTGATGAATGC

1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA  
CCATGTTCAGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT

1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC  
TGACTACGGTGGGCGACCGTTTATACCCTATTACCTTGGTAGTATTTAGG

1251 CAGATCTAGTCTAGTTTTAGCAGCGACATCAGGGAACAGTGGTACCACAC  
GTCTAGATCAGATCAAAATCGTCGCTGTAGTCCCTTGTCACCATGGTGTG

1301 TTACAGTGCAAACCAACATTTATGCCGTTAGTCAAGGTTGGCTTCCTACT  
AATGTCACGTTTGGTTGTAAATACGGCAATCAGTTCCAACCGAAGGATGA

1351 AATAATACACAACCTTTTGTACAACCATGTTGGGCTATATGGTCTGTG  
TTATTATGTGTTGGAAAACAATGTTGGTAACAACCCGATATACCAGACAC

1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA  
GAACGTTTCGTTTATCACCTGTTTACCTATCTCCTGACATCGTCACTTT

1451 AGGCTGAACAACAGTGGGCTCTTTATGCAGATGGTTCAATACGTCCTCAG  
TCCGACTTGTGTGCACCCGAGAAATACGTCCTACCAAGTTATGCAGGAGTC

1501 CAAAACCGAGATAATTGCCCTTACAAGTGATTCTAATATACGGGAAACAGT  
GTTTTGGCTCTATTAAACGGAATGTTCACTAAGATTATATGCCCTTTGTCA

1551 TGTTAAGATCCTCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT  
ACAATTCTAGGAGAGAACACCGGGACGTAGGAGACCGGTTGCTACCTACA

1601 TCAAGAATGATGGAACCATTTTAAATTTGTATAGTGGATTGGTGTAGAT  
AGTTCTTACTACCTTGGTAAAATTTAAACATATCACCTAACCACAATCTA

1651 GTGAGGCGATCGGATCCGAGCCTTAAACAAATCATTCTTTACCCTCTCCA  
CACTCCGCTAGCCTAGGCTCGGAATTTGTTTAGTAAGAAATGGGAGAGGT

1701 TGGTGACCCAAACCAATATGGTTACCATTATTTTGTATAGACAGATTACT  
ACCACTGGGTTTGGTTTATACCAATGGTAATAAACTATCTGTCTAATGA

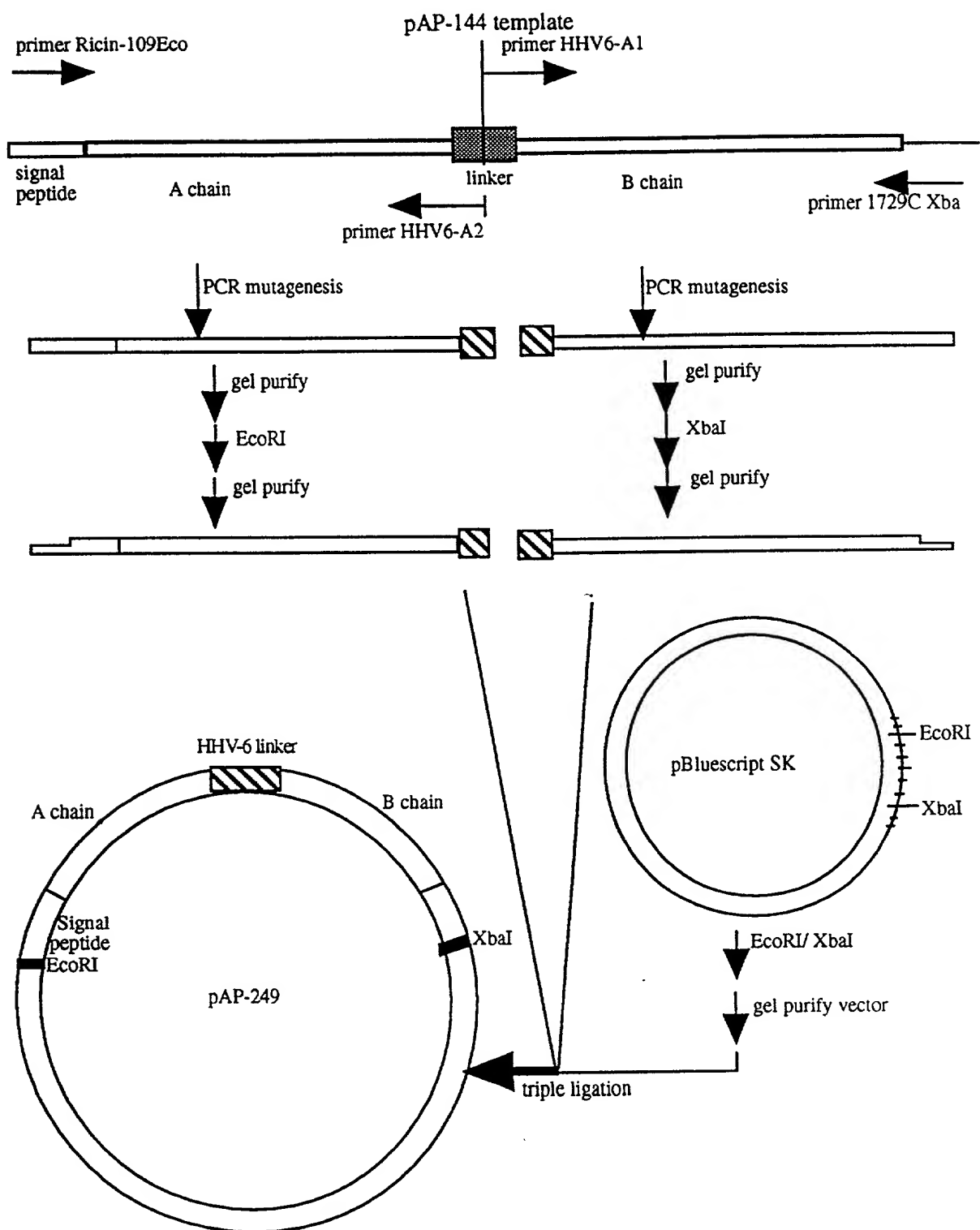
1751 CTCTTGCAAGTGTGTGTGCTGCCATGAAAATAGATGGCTTAAATAAAAA  
GAGAACGTCACACACACAGGACGGTACTTTTATCTACCGAATTTATTTTT

1801 GGACATTGTAAATTTTGTAACTGAAAGGACAGCAAGTTATATCGAATTCC  
CCTGTAACATTTAAACATTGACTTTTCTGTCGTTCAATATAGCTTAAGG

1851 TGCAG  
ACGTC

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**FIGURE 20A**



# FIGURE 20B

## WT preproricin linker

primer HHV6-A1

5' - TCGGTGCCAAATTTAAT -3'

TCCTTGCTTATAAGGCCAGTGGTGCCAAATTTAAT  
AGAAACGAAATATTCGGTCACCCACGGTTAAAAATTA

3' - AGCAGTGTCAAAAGAGCTAAATTTACGT-5'

primer HHV6-A2

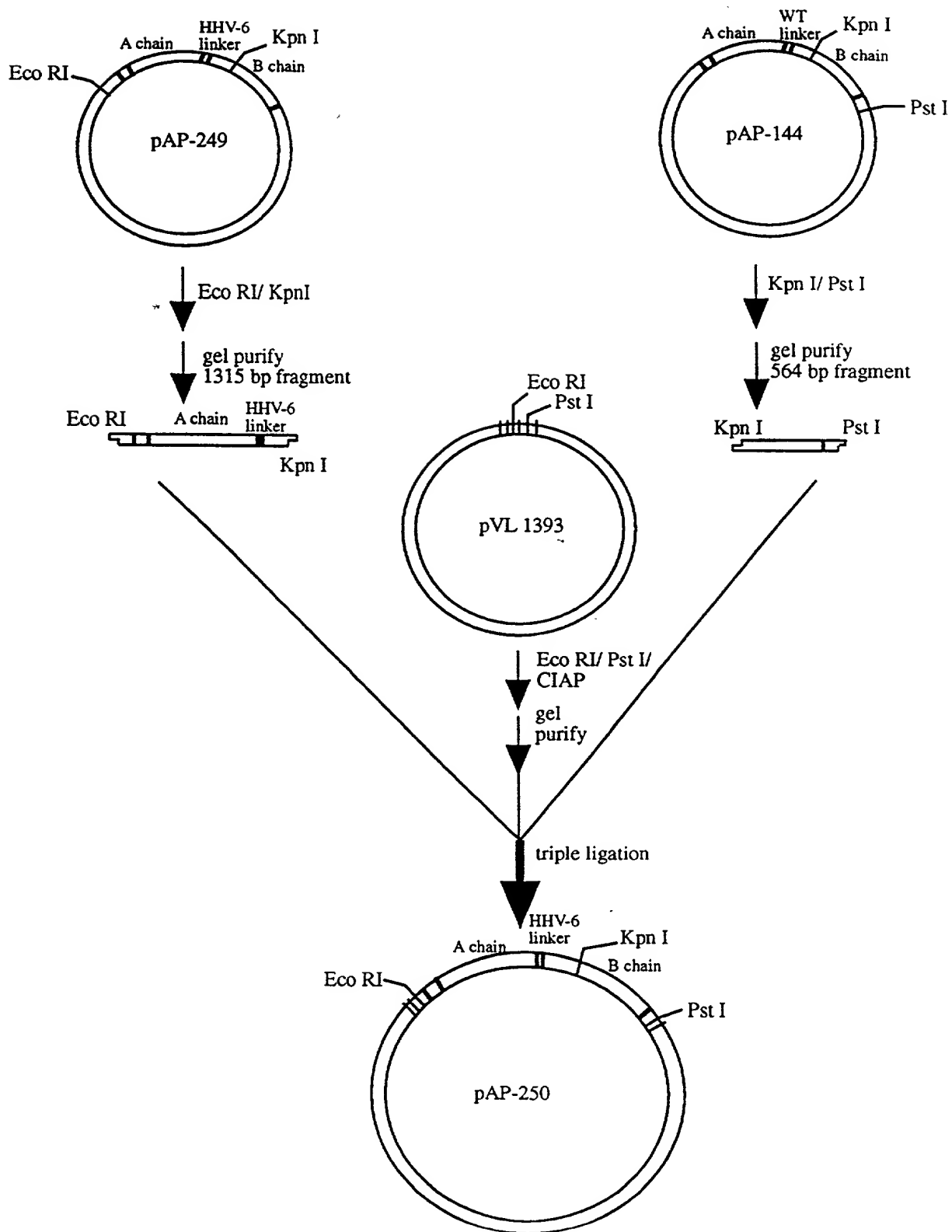
PCR mutagenesis  
ligate with pBluescript SK

pAP 249 linker  
(HHV-6 variant)

TCCTCGATTTTAAATGCATCGGTGCCAAATTTAAT  
AGAAAGCTAAATTTACGTAGCCACGGTTAAAAATTA

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**FIGURE 20C**



00440" F5 F560

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## FIGURE 20D

10 20 30 40 50

1 GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT  
CTTAAGTACTTTGGCCCTCCTTTATGATAACATTATACCTACATACGTCA

51 GGCAACATGGCTTTGTTTTGGATCCACCTCAGGGTGGTCTTTCACATTAG  
CCGTTGTACCGAAACAAAACCTAGGTGGAGTCCCACCAGAAAGTGTAAATC

101 AGGATAACAACATATTCCCCAAACAATACCCAATTATAAACTTTACCACA  
TCCTATTGTTGTATAAGGGGTTTGTATGGGTAAATATTTGAAATGGTGT

151 GCGGGTGCCACTGTGCAAAAGCTACACAACTTTATCAGAGCTGTTTCGCGG  
CGCCACGGTGACACGTTTCGATGTGTTGAAATAGTCTCGACAAGCGCC

201 TCGTTTAACAACTGGAGCTGATGTGAGACATGATATACCAGTGTGCCAA  
AGCAAATTGTTGACCTCGACTACACTCTGTACTATATGGTCACAACGGTT

251 ACAGAGTTGGTTTGCCTATAAACCAACGGTTTATTTTAGTTGAACTCTCA  
TGTCTCAACCAAACGATATTTGGTTGCCAAATAAAATCAACTTGAGAGT

301 AATCATGCAGAGCTTCTGTTACATTAGCGCTGGATGTCACCAATGCATA  
TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT

351 TGTGGTTCGGCTACCGTGTGGAATAGCGCATATTTCTTTCATCCTGACA  
ACACCAGCCGATGGCACGACCTTTATCGCGTATAAAGAAAGTAGGACTGT

401 ATCAGGAAGATGCAGAAGCAATCACTCATCTTTTCACTGATGTTCAAAAT  
TAGTCCTTCTACGTCTTCGTTAGTGAGTAGAAAAGTGACTACAAGTTTTA

451 CGATATACATTTCGCCCTTTGGTGGTAATTATGATAGACTTGAACAACCTGC  
GCTATATGTAAGCGGAAACCACCATTAATACTATCTGAACCTGTTGAACG

501 TGGTAATCTGAGAGAAAATATCGAGTTGGGAAATGGTCCACTAGAGGAGG  
ACCATTAGACTCTCTTTTATAGCTCAACCCTTTACCAGGTGATCTCCTCC

551 CTATCTCAGCGCTTTATTATTACAGTACTGGTGGCACTCAGCTTCCAAC  
GATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA

601 CTGGCTCGTTCCTTTATAATTTGCATCCAATGATTTCAGAAGCAGCAAG  
GACCGAGCAAGGAAATATTAAACGTAGGTTTACTAAAGTCTTCGTCGTTT

651 ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA  
TAAGGTTATATAACTCCCTCTTTACGCGTGCTCTTAATCCATGTTGGCCT

701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGGAGA  
CTAGACGTGGTCTAGGATCGCATTAAATGTGAACCTTATCAACCCCTCT

751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTTGCTAGTCCAAT  
GAAAGGTGACGTTAAGTTCTCAGATTGGTTTCTCGGAAACGATCAGGTTA

801 TCAACTGCAAAGACGTAATGGTTCCAAATTCAGTGTGTACGATGTGAGTA  
AGTTGACGTTTCTGCATTACCAAGGTTTAAAGTCACACATGCTACACTCAT

851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGCACCTCCACCA  
ATAATTAGGGATAGTATCGAGAGTACCACATATCTACGCGTGGAGGTGGT

901 TCGTCACAGTTTCTTCGATTTTAAATGCATCGGTGCCAAATTTTAATGC  
AGCAGTGTCAAAAGAAGCTAAAATTTACGTAGCCACGGTTTAAAATTACG

**FIGURE 20D (CONT'D)**

951 TGATGTTTGTATGGATCCTGAGCCCATAGTGCGTATCGTAGGTCGAAATG  
 ACTACAAACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTTAC  
 1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA  
 CAGATACACAACATAATCCCTACCTTCTAAGGTGTTGCCTTTGCGTTAT  
 1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT  
 GTCAACACCGGTACGTTTACGATTATGTCTACGTTTAGTCGAGACCTGAAA  
 1101 GAAAAGAGACAATACTATTTCGATCTAATGGAAAGTGTTTAACTACTTACG  
 CTTTTCTCTGTTATGATAAGCTAGATTACCTTTCACAAATTGATGAATGC  
 1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA  
 CCATGTCAGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT  
 1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC  
 TGACTACGGTGGGCGACCGTTTATACCTATTACCTTGGTAGTATTTAGG  
 1251 CAGATCTAGTCTAGTTTTAGCAGCGACATCAGGGAACAGTGGTACCACAC  
 GTCTAGATCAGATCAAAATCGTCGCTGTAGTCCCTTGTCAACCATGGTGTG  
 1301 TTACAGTGCAAACCAACATTTATGCCGTTAGTCAAGGTTGGCTTCCTACT  
 AATGTCACGTTTGGTTGTAAATACGGCAATCAGTTCCAACCGAAGGATGA  
 1351 AATAATACACAACCTTTTGTGTTACAACCATTTGTTGGGCTATATGGTCTGTG  
 TTATTATGTGTTGGAAAACAATGTTGGTAACAACCCGATATACCAGACAC  
 1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA  
 GAACGTTTCGTTTATCACCTGTTTCATACCTATCTCCTGACATCGTCACTTT  
 1451 AGGCTGAACAACAGTGGGCTCTTTATGCAGATGGTTCAATACGTCCTCAG  
 TCCGACTTGTGTGTCACCCGAGAAATACGTCCTACCAAGTTATGCAGGAGTC  
 1501 CAAAACCGAGATAATTGCCCTTACAAGTGATTCTAATATACGGGAAACAGT  
 GTTTTGGCTCTATTAACGGAATGTTCACTAAGATTATATGCCCTTTGTCA  
 1551 TGTTAAGATCCTCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT  
 ACAATTCTAGGAGAGAACACCGGGACGTAGGAGACCGGTTGCTACCTACA  
 1601 TCAAGAATGATGGAACCATTTTAAATTTGTATAGTGGATTGGTGTTAGAT  
 AGTTCTTACTACCTTGGTAAAATTTAAACATATCACCTAACCAATCTA  
 1651 GTGAGGCGATCGGATCCGAGCCTTAAACAAATCATTCTTTACCCTCTCCA  
 CACTCCGCTAGCCTAGGCTCGGAATTTGTTTAGTAAGAAATGGGAGAGGT  
 1701 TGGTGACCCAAACCAATATGGTTACCATTATTTTGATAGACAGATTACT  
 ACCACTGGGTTTGGTTTATACCAATGGTAATAAACTATCTGTCTAATGA  
 1751 CTCTTGCAGTGTGTGTCTGTCCTGCCATGAAAATAGATGGCTTAAATAAAAA  
 GAGAACGTCACACACACAGGACGGTACTTTTATCTACCGAATTTATTTTT  
 1801 GGACATTGTAAATTTTGTAACTGAAAGGACAGCAAGTTATATCGAATTCC  
 CCTGTAACATTTAAACATTGACTTTCCTGTCGTTCAATATAGCTTAAGG  
 1851 TGCAG  
 ACGTC

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## FIGURE 21

Ricin linker (wild type):

A chain- S L L I R P V V P N F N -B chain

pAP-213/pAP-214 linker (Cathepsin B):

A chain- S L L K S R M V P N F N -B chain

pAP-215/pAP-216 linker (MMP-3):

A chain- R P K P Q Q F F G L M N -B chain

pAP-217/pAP-218 linker (MMP-7):

A chain- S L R P L A L W R S F N -B chain

pAP-219/pAP-220 linker (MMP-9):

A chain- S P Q G I A G Q R N F N -B chain

pAP-221/pAP-222 linker (THERMOLYSIN-LIKE MMP):

A chain- D V D E R D V R G F A S F L -B chain

pAP-241/pAP-242 linker (EBV-A):

A chain- S K L V Q A S A S G V N -B chain

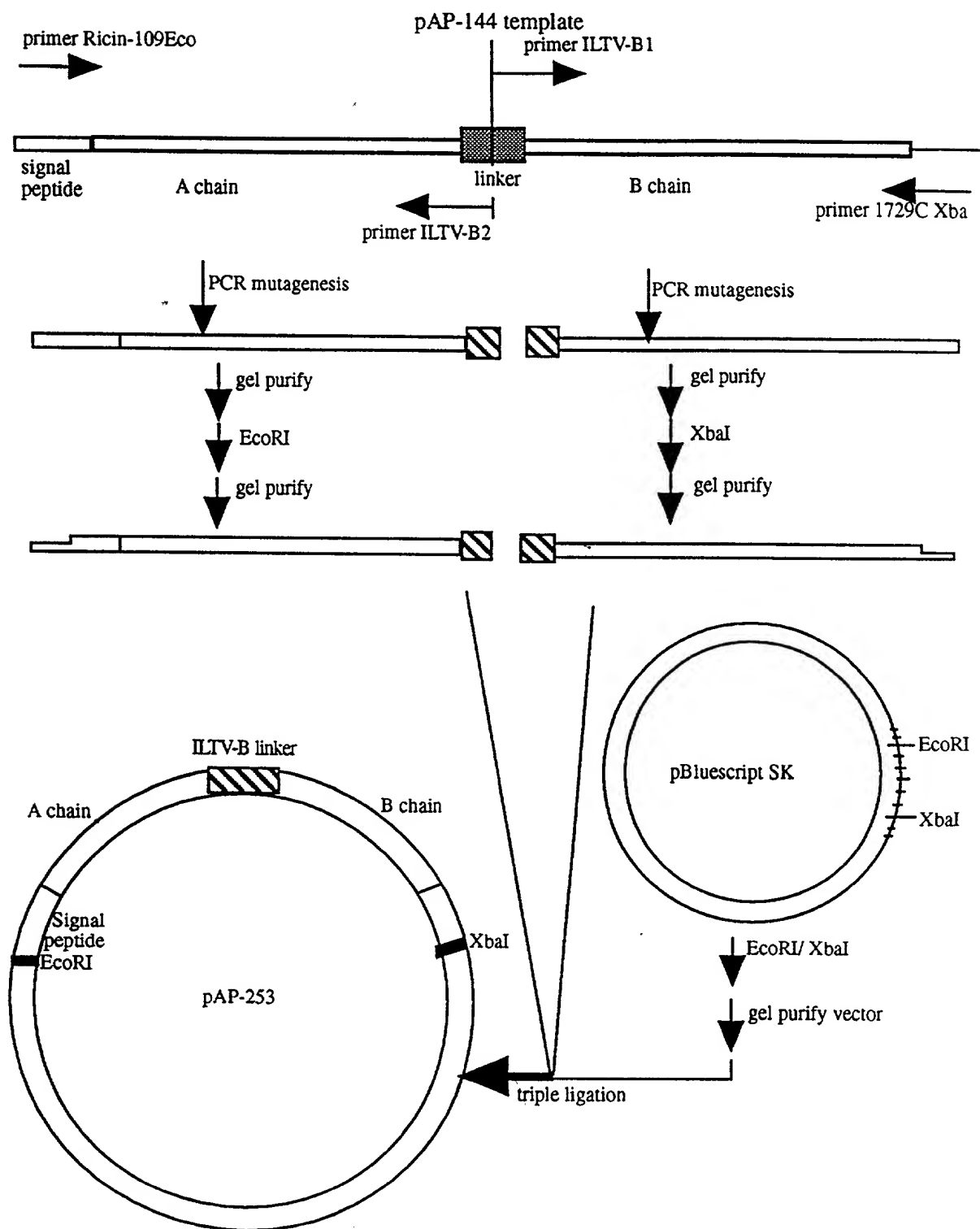
pAP-243/pAP-244 linker (EBV-B):

A chain- S S Y L K A S D A P D N -B chain

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**FIGURE 22A**





## FIGURE 22B

### WT preproricin linker

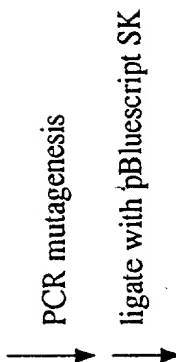
primer ILTV-B1

5' - AATGAGGTAATTACTAATGCTGATGTTTGT -3'

—————TCTTTGCTTATAAGGCCAGTGGTGCCAAATTTTAAT—————  
 —————AGAAACGAATATTCCCGT|CACCACGGTTTAAATTA—————

3' - AGCAGTGTCAAAGATTTCATAGATGTCCTCGT-5'

primer ILTV-B2

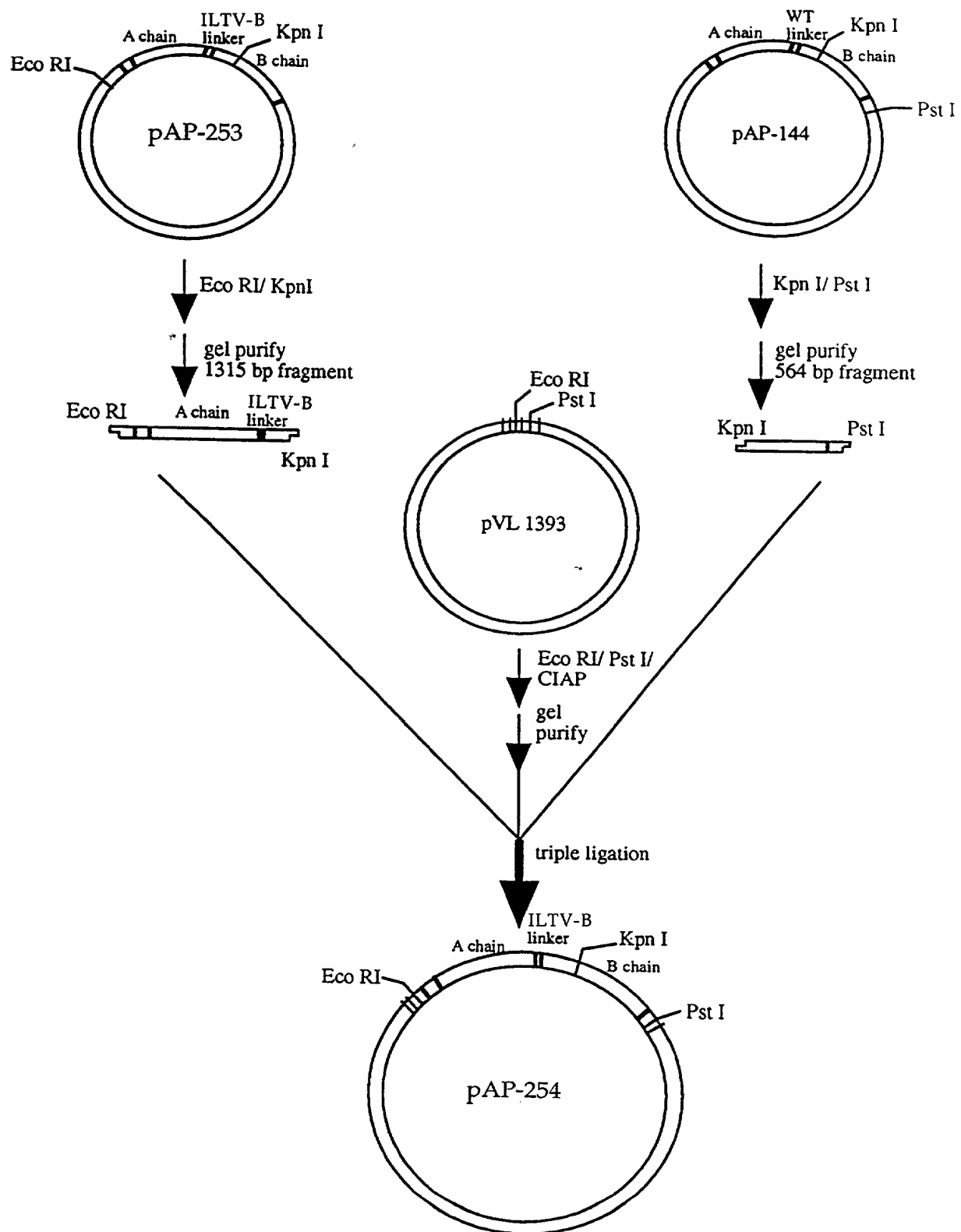


pAP 253 linker  
(ILTV-B variant)

—————TCTAAGTATCTACAGGCAAAATGAGGTAATTACTAAT—————  
 —————AGATTCATAGATGTCCTCGTTACTCCCATTAATGATTA—————

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**FIGURE 22C**



**FIGURE 22D**

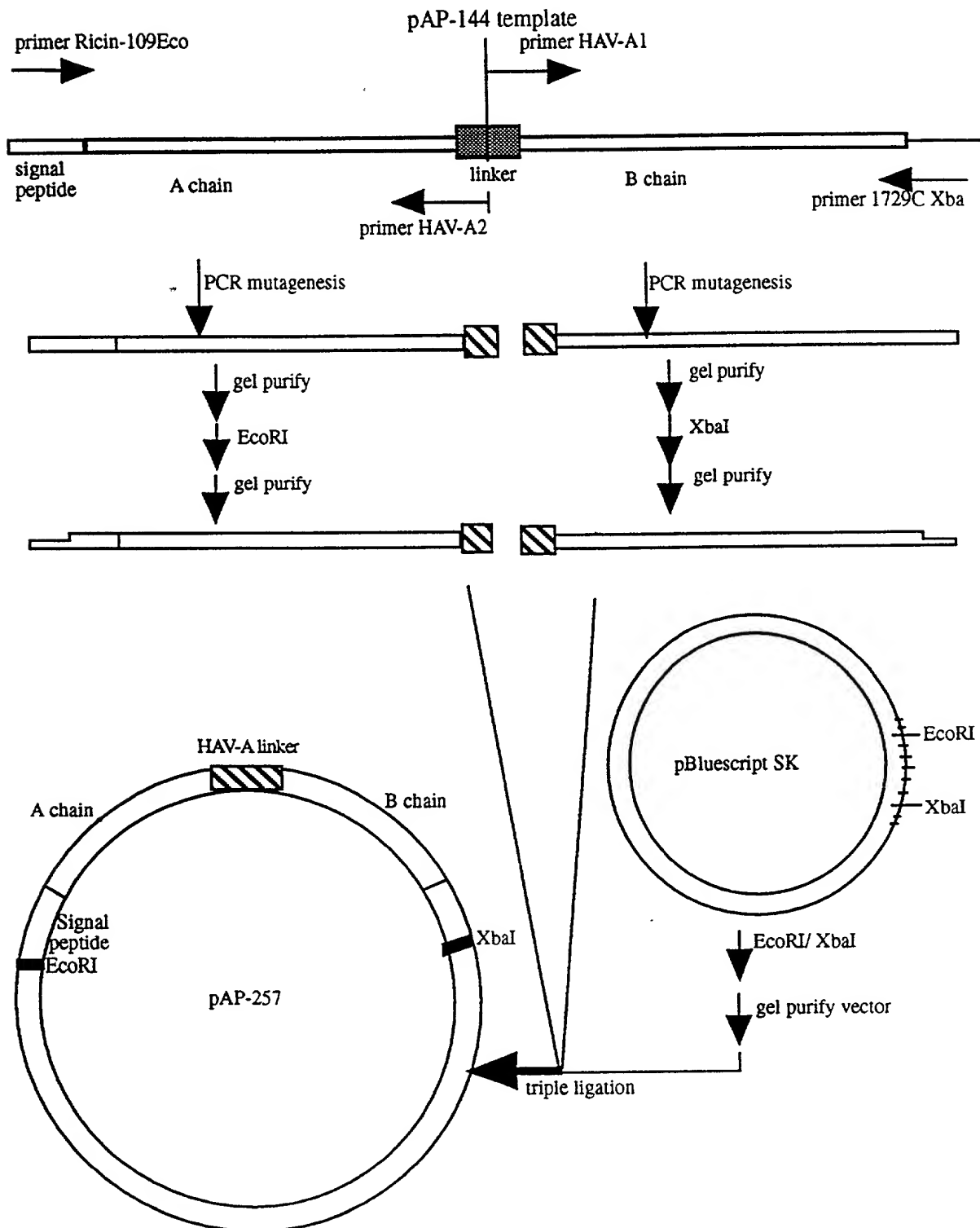
[illegible]

**FIGURE 22D (CONT'D)**

951 TGATGTTTGTATGGATCCTGAGCCCATAGTGCGTATCGTAGGTCGAAATG  
 ACTACAAACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTTAC  
 1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA  
 CAGATACACAACCTACAATCCCTACCTTCTAAGGTGTTGCCTTTGCGTTAT  
 1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT  
 GTCAACACCGGTACGTTTACGATTATGTCTACGTTTAGTCGAGACCTGAAA  
 1101 GAAAAGAGACAATACTATTTCGATCTAATGGAAAGTGTTTAACTACTTACG  
 CTTTTCTCTGTTATGATAAGCTAGATTACCTTTCACAAATTGATGAATGC  
 1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA  
 CCATGTCAGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT  
 1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC  
 TGACTACGGTGGGCGACCGTTTATACCCTATTACCTTGGTAGTATTTAGG  
 1251 CAGATCTAGTCTAGTTTTAGCAGCGACATCAGGGAACAGTGGTACCACAC  
 GTCTAGATCAGATCAAAATCGTCGCTGTAGTCCCTTGTCACCATGGTGTG  
 1301 TTACAGTGCAAACCAACATTTATGCCGTTAGTCAAGGTTGGCTTCCTACT  
 AATGTTCACGTTTGGTTGTAAATACGGCAATCAGTTCCAACCGAAGGATGA  
 1351 AATAATACACAACCTTTTGTACAAACCATTGTTGGGCTATATGGTCTGTG  
 TTATTATGTGTTGGAACAATGTTGGTAACAACCCGATATACCAGACAC  
 1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA  
 GAACGTTTCGTTTATCACCTGTTTCATACCTATCTCCTGACATCGTCACTTT  
 1451 AGGCTGAACAACAGTGGGCTCTTTATGCAGATGGTTCAATACGTCCTCAG  
 TCCGACTTGTTGTCAACCGAGAAATACGTCTACCAAGTTATGCAGGAGTC  
 1501 CAAAACCGAGATAATTGCCTTACAAGTGATTCTAATATACGGGAAACAGT  
 GTTTTGGCTCTATTACGGAATGTTCACTAAGATTATATGCCCTTTGTCA  
 1551 TGTTAAGATCCTCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT  
 ACAATTCTAGGAGAGAACACCGGGACGTAGGAGACCGGTTGCTACCTACA  
 1601 TCAAGAATGATGGAACCATTTTAAATTTGTATAGTGGATTGGTGTAGAT  
 AGTTCTTACTACCTTGGTAAATTTAAACATATCACCTAACCACAATCTA  
 1651 GTGAGGCGATCGGATCCGAGCCTTAAACAAATCATTCTTTACCTCTCCA  
 CACTCCGCTAGCCTAGGCTCGGAATTTGTTTAGTAAGAAATGGGAGAGGT  
 1701 TGGTGACCCAAACCAATATGGTTACCATTATTTTGATAGACAGATTACT  
 ACCACTGGGTTTGGTTTATACCAATGGTAATAAACTATCTGTCTAATGA  
 1751 CTCTTGCAAGTGTGTGTGCTGCGCATGAAAATAGATGGCTTAAATAAAAA  
 GAGAACGTCACACACACAGGACGGTACTTTTATCTACCGAATTTATTTTTT  
 1801 GGACATTGTAAATTTTGTAACTGAAAGGACAGCAAGTTATATCGAATTCC  
 CCTGTAACATTTAAACATTGACTTTTCTGTGTTCAATATAGCTTAAGG  
 1851 TGCAG  
 ACGTC

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**FIGURE 23A**



# FIGURE 23B

## WT preprorcin linker

primer HAV-A1

5' - TCGTTCCTCAAAATTGGAATGCTGATGTTTGT -3'

TCTTTCCTTATAAGGCCAGTGGTGCCAAATTTTAAT  
AGAAACGAATATTCCTGGT\*  
CACCACGGTTTAAATTA

3' - AGCAGTGTCAAAAGACTCGAATCTTTCGGT -5'

primer HAV-A2

PCR mutagenesis

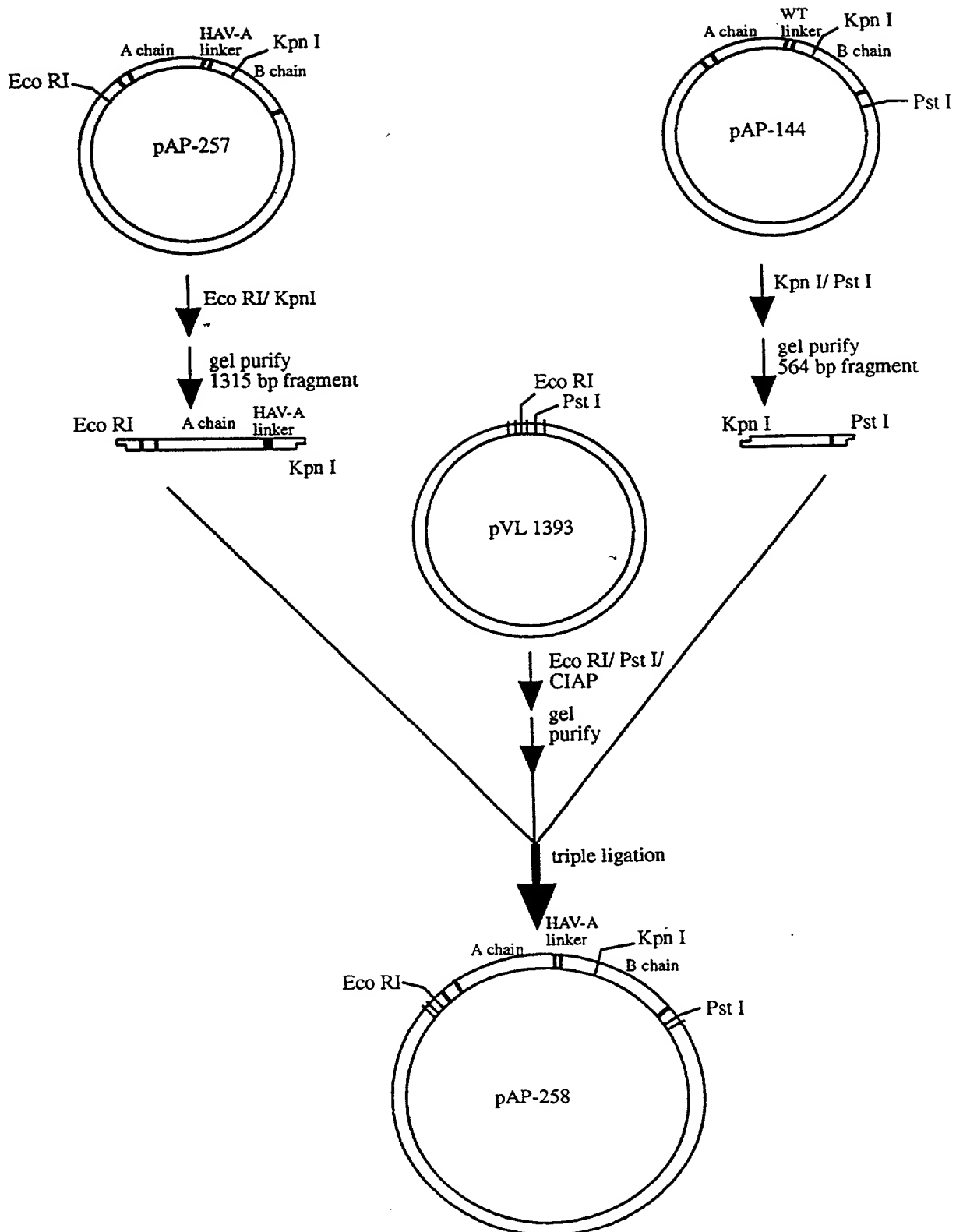
ligate with pBluescript SK

pAP 257 linker  
(HAV-A variant)

TCTGAGCTTAGAACGCAATCGTTCCTCAAAATTGGAAT  
AGACTCGAATCTTTCGGTTAGCAAGAGTTAACCTTA

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**FIGURE 23C**



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# FIGURE 23D

	10	20	30	40	50
1	GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT				
	CTTAAGTACTTTGGCCCTCCTTTATGATAACATTATACCTACATACGTCA				
51	GGCAACATGGCTTTGTTTTGGATCCACCTCAGGGTGGTCTTTCACATTAG				
	CCGTTGTACCGAAACAAAACCTAGGTGGAGTCCCACCAGAAAGTGTAATC				
101	AGGATAACAACATATTCCCCAAACAATACCCAATTATAAACTTTACCACA				
	TCCTATTGTTGTATAAGGGGTTTGTATGGGTTAATATTTGAAATGGTGT				
151	GCGGGTGCCACTGTGCAAAGCTACACAACTTTATCAGAGCTGTTTCGCGG				
	CGCCACGGTGACACGTTTCGATGTGTTTGAAATAGTCTCGACAAGCGCC				
201	TCGTTTAACTGAGCTGATGTGAGACATGATATACCAGTGTTCGCCAA				
	AGCAAATTGTTGACCTCGACTACACTCTGTACTATATGGTCACAACGGTT				
251	ACAGAGTTGGTTTGCTTATAAACCACCGGTTTATTTTAGTTGAACTCTCA				
	TGTCTCAACCAAACGGATATTTGGTTGCCAAATAAAATCAACTTGAGAGT				
301	AATCATGCAGAGCTTCTGTTACATTAGCGCTGGATGTCACCAATGCATA				
	TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT				
351	TGTGGTCGGCTACCGTGCTGGAAATAGCGCATATTTCTTTCATCCTGACA				
	ACACCAGCCGATGGCAGACCTTTATCGCGTATAAAGAAAGTAGGACTGT				
401	ATCAGGAAGATGCAGAAGCAATCACTCATCTTTTCACTGATGTTCAAAT				
	TAGTCCTTCTACGTCTTCGTTAGTGAGTAGAAAAGTGACTACAAGTTTTA				
451	CGATATACATTTCGCCCTTTGGTGGTAATTATGATAGACTTGAACAACCTGC				
	GCTATATGTAAGCGGAAACCACCATTAATACTATCTGAACCTGTTGAACG				
501	TGGTAATCTGAGAGAAAATATCGAGTTGGGAAATGGTCCACTAGAGGAGG				
	ACCATTAGACTCTCTTTTATAGCTCAACCCTTTACCAGGTGATCTCCTCC				
551	CTATCTCAGCGCTTTATTATTACAGTACTGGTGGCACTCAGCTTCCAAC				
	GATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA				
601	CTGGCTCGTTTCCTTTATAATTTGCATCCAAATGATTTTCAAGCAGCAAG				
	GACCGAGCAAGGAAATATTAAACGTAGGTTTACTAAAGTCTTCGTCGTTT				
651	ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA				
	TAAGGTTATATAACTCCCTCTTTACGCGTGCTCTTAATCCATGTTGGCCT				
701	GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGGAGA				
	CTAGACGTGGTCTAGGATCGCATTAATGTGAACTCTTATCAACCCCTCT				
751	CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTTGCTAGTCCAAT				
	GAAAGGTGACGTTAAGTTCTCAGATTGGTTTCCTCGGAAACGATCAGGTTA				
801	TCAACTGCAAAGACGTAATGGTTCCAAATTCAGTGTGTACGATGTGAGTA				
	AGTTGACGTTTCTGCATTACCAAGGTTTAAGTCACACATGCTACACTCAT				
851	TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGCACCTCCACCA				
	ATAATTAGGGATAGTATCGAGAGTACCACATATCTACGCGTGGAGGTGGT				
901	TCGTACAGTTTTCTGAGCTTAGAACGCAATCGTTCTCAAATTGGAATGC				
	AGCAGTGTCAAAAGACTCGAATCTTGCGTTAGCAAGAGTTTAACCTTACG				

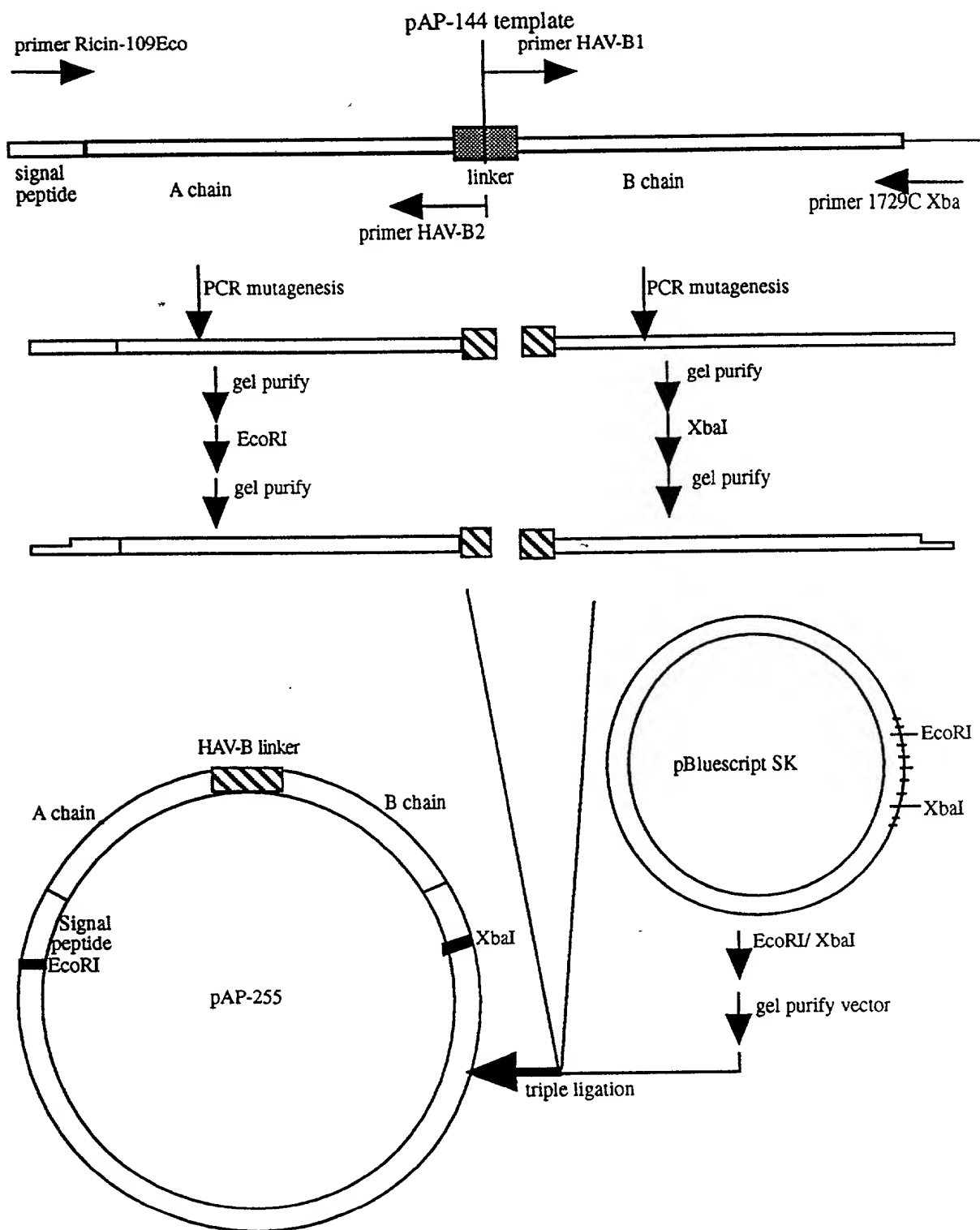


**FIGURE 23D (CONT'D)**

951 TGATGTTTGTATGGATCCTGAGCCCATAGTGCGTATCGTAGGTCGAAATG  
 ACTACAAACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTTAC  
 1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA  
 CAGATACACAACATAATCCCTACCTTCTAAGGTGTTGCCTTTGCGTTAT  
 1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT  
 GTCAACACCGGTACGTTTACGATTATGTCTACGTTTAGTCGAGACCTGAAA  
 1101 GAAAAGAGACAATACTATTCGATCTAATGGAAAGTGTTTAACTACTTACG  
 CTTTTCTCTGTTATGATAAGCTAGATTACCTTTCACAAATTGATGAATGC  
 1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA  
 CCATGTCAGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT  
 1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC  
 TGACTACGGTGGGCGACCGTTTATACCCTATTACCTTGGTAGTATTTAGG  
 1251 CAGATCTAGTCTAGTTTTAGCAGCGACATCAGGGAACAGTGGTACCACAC  
 GTCTAGATCAGATCAAAATCGTCGCTGTAGTCCCTTGTCAACCATGGTGTG  
 1301 TTACAGTGCAAACCAACATTTATGCCGTTAGTCAAGGTTGGCTTCCTACT  
 AATGTACGTTTGGTTGTAAATACGGCAATCAGTTCCAACCGAAGGATGA  
 1351 AATAATACACAACCTTTTGTTACAACCATTGTTGGGCTATATGGTCTGTG  
 TTATTATGTGTTGGAAAACAATGTTGGTAACAACCCGATATACCAGACAC  
 1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA  
 GAACGTTTCGTTTATCACCTGTTTCATACCTATCTCCTGACATCGTCACTTT  
 1451 AGGCTGAACAACAGTGGGCTCTTTATGCAGATGGTTCAATACGTCCTCAG  
 TCCGACTTGTGTGTCACCCGAGAAATACGTCTACCAAGTTATGCAGGAGTC  
 1501 CAAAACCGAGATAATTGCCTTACAAGTGATTCTAATATACGGGAACAGT  
 GTTTTGGCTCTATTAACGGAATGTTCACTAAGATTATATGCCCTTTGTCA  
 1551 TGTTAAGATCCTCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT  
 ACAATTCTAGGAGAGAAACACCGGGACGTAGGAGACCGGTTGCTACCTACA  
 1601 TCAAGAATGATGGAACCATTTTAAATTTGTATAGTGGATTGGTGTTAGAT  
 AGTTCTTACTACCTTGGTAAAAATTTAAACATATCACCTAACCACAATCTA  
 1651 GTGAGGCGATCGGATCCGAGCCTTAAACAAATCATTCTTTACCCCTCTCCA  
 CACTCCGCTAGCCTAGGCTCGGAATTTGTTTAGTAAGAAATGGGAGAGGT  
 1701 TGGTGACCCAAACCAAATATGGTTACCATTATTTTGATAGACAGATTACT  
 ACCACTGGGTTTGGTTTATACCAATGGTAATAAACTATCTGTCTAATGA  
 1751 CTCTTGCAGTGTGTGTGTCTGCGCATGAAAATAGATGGCTTAAATAAAAA  
 GAGAACGTCACACACACAGGACGGTACTTTTATCTACCGAATTTATTTTT  
 1801 GGACATTGTAAATTTTGTAACTGAAAGGACAGCAAGTTATATCGAATTCC  
 CCTGTAACATTTAAACATTGACTTTCCTGTCGTTCAATATAGCTTAAGG  
 1851 TGCAG  
 ACGTC

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**FIGURE 24A**



004440"TSF560

## FIGURE 24B

WT preprorin linker

primer HAV-B1

5' - GGGATCGATGATGATAATGCTGATGTTGT -3'

TCTTTCCTTATAAGGCCAGTGGTGCCAAATTTAAT  
AGAAACGAAATATTCCTGGTCAACACGGTTAAATA

3' - AGCAGTGTCAAAAGACTCGAAACACGCGTT-5'

primer HAV-B2

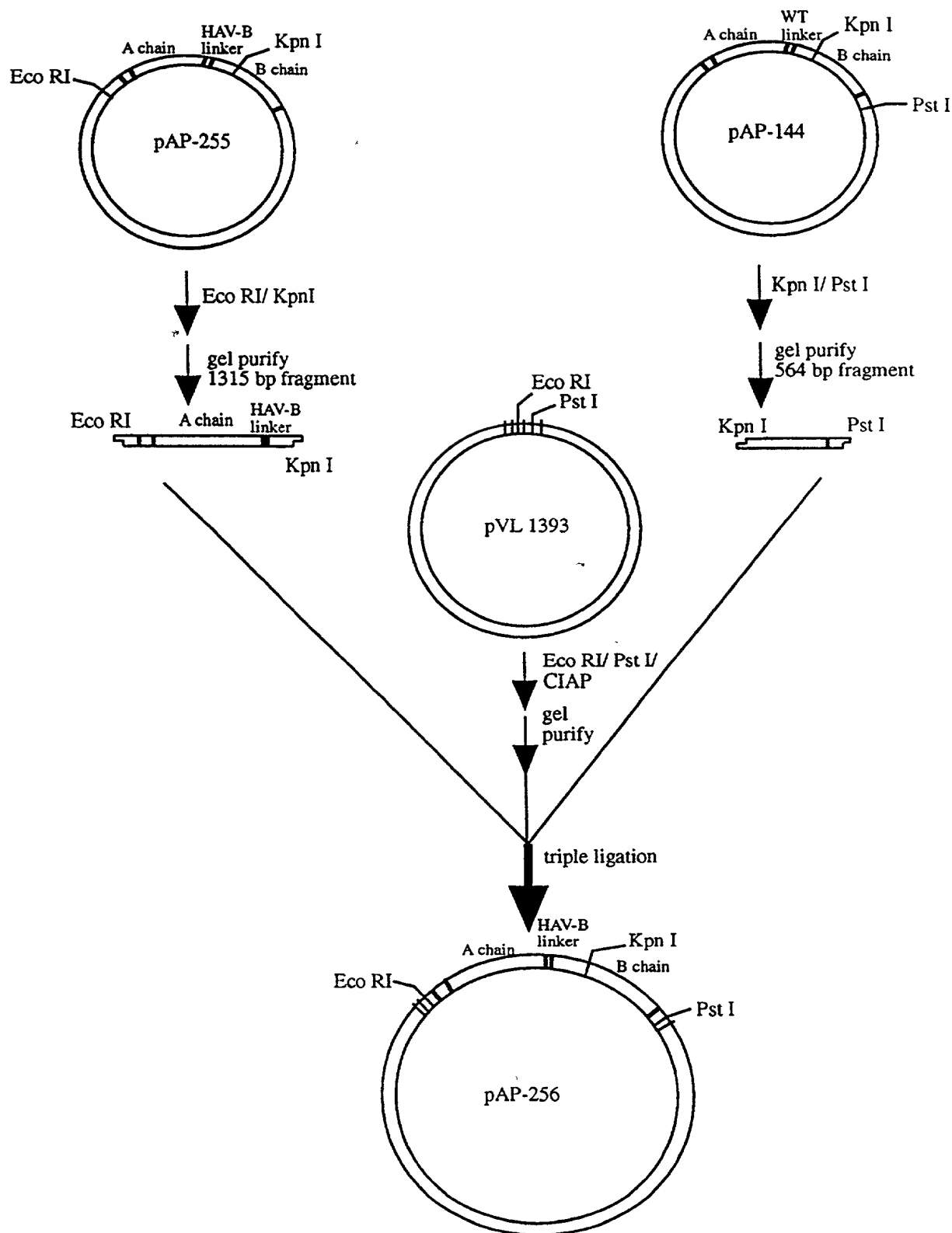
PCR mutagenesis  
ligate with pBluescript SK

pAP 255 linker  
(HAV-B variant)

TCTGAGCTTTGGTCGCAAGGATCGATGATAAT  
AGACTCGAAACACGCGTTCCCTAGCTACTATTA

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**FIGURE 24C**



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**FIGURE 24D**

10 20 30 40 50

1 GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT  
CTTAAGTACTTTGGCCCTCCTTTATGATAACATTATACCTACATACGTCA

51 GGCAACATGGCTTTGTTTGGATCCACCTCAGGGTGGTCTTTCACATTAG  
CCGTTGTACCGAAACAAAACCTAGGTGGAGTCCCACCAGAAAGTGTAATC

101 AGGATAACAACATATTCCCCAAACAATACCCAATTATAAACTTTACCACA  
TCCTATTGTTGTATAAGGGGTTTGTATGGGTTAATATTTGAAATGGTGT

151 GCGGGTGCCACTGTGCAAAGCTACACAACTTTATCAGAGCTGTTGCGGG  
CGCCCACGGTGACAGTTTCGATGTGTTGAAATAGTCTCGACAAGCGCC

201 TCGTTTAAACAACTGGAGCTGATGTGAGACATGATATACCAGTGTGCCAA  
AGCAAATTGTTGACCTCGACTACACTCTGTACTATATGGTCACAACGGTT

251 ACAGAGTTGGTTTGCCTATAAACCAACGGTTTATTTTAGTTGAACTCTCA  
TGTCTCAACCAACGGATATTTGGTTGCCAAATAAAATCAACTTGAGAGT

301 AATCATGCAGAGCTTCTGTTACATTAGCGCTGGATGTCACCAATGCATA  
TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT

351 TGTGGTCGGCTACCGTGCTGGAAATAGCGCATATTTCTTTCATCCTGACA  
ACACCAGCCGATGGCAGGACCTTTATCGCGTATAAAGAAAGTAGGACTGT

401 ATCAGGAAGATGCAGAAGCAATCACTCATCTTTTCACTGATGTTCAAAAT  
TAGTCCTTCTACGTCTTCGTTAGTGAGTAGAAAAGTGACTACAAGTTTTA

451 CGATATACATTCGCCCTTTGGTGGTAATTATGATAGACTTGAACAACTTGC  
GCTATATGTAAGCGGAAACCACCATTAATACTATCTGAACTTGTGTAACG

501 TGGTAATCTGAGAGAAAATATCGAGTTGGGAAATGGTCCACTAGAGGAGG  
ACCATTAGACTCTCTTTTATAGCTCAACCCTTTACCAGGTGATCTCCTCC

551 CTATCTCAGCGCTTTATTATTACAGTACTGGTGGCACTCAGCTTCCAAC  
GATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA

601 CTGGCTCGTTCCTTTATAATTTGCATCCAAATGATTTCAGAAGCAGCAAG  
GACCGAGCAAGGAAATATTAAACGTAGGTTTACTAAAGTCTTCGTCGTTT

651 ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA  
TAAGGTTATATAACTCCCTCTTTACGCGTGCTCTTAATCCATGTTGGCCT

701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGGAGA  
CTAGACGTGGTCTAGGATCGCATTAAATGTGAACTCTTATCAACCCCTCT

751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTTGCTAGTCCAAT  
GAAAGGTGACGTTAAGTTCTCAGATTGGTTCCCTCGGAAACGATCAGGTTA

801 TCAACTGCAAAGACGTAATGGTTCCAAATTCAGTGTGTACGATGTGAGTA  
AGTTGACGTTTCTGCATTACCAAGGTTTAAGTCACACATGCTACACTCAT

851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGCACCTCCACCA  
ATAATTAGGGATAGTATCGAGAGTACCACATATCTACGCGTGGAGGTGGT

901 TCGTCACAGTTTCTGAGCTTTGGTCGCAAGGGATCGATGATGATAATGC  
AGCAGTGTCAAAAGACTCGAAACCAGCGTTCCCTAGCTACTACTATTACG

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## FIGURE 24D (CONT'D)

951 TGATGTTTGTATGGATCCTGAGCCCATAGTGCGTATCGTAGGTCGAAATG  
ACTACAAACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTTAC

1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA  
CAGATACACAACATAATCCCTACCTTCTAAGGTGTTGCCTTTGCGTTAT

1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT  
GTCAACACCGGTACGTTTCAGATTATGTCTACGTTTAGTCGAGACCTGAAA

1101 GAAAAGAGACAATACTATTTCGATCTAATGGAAAGTGTTTAACTACTTACG  
CTTTTCTCTGTTATGATAAGCTAGATTACCTTTCACAAATTGATGAATGC

1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA  
CCATGTACAGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT

1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC  
TGACTACGGTGGGCGACCGTTTATACCCTATTACCTTGGTAGTATTTAGG

1251 CAGATCTAGTCTAGTTTTAGCAGCGACATCAGGGAACAGTGGTACCACAC  
GTCTAGATCAGATCAAAATCGTCGCTGTAGTCCCTTGTCAACCATGGTGTG

1301 TTACAGTGCAAACCAACATTTATGCCGTTAGTCAAGGTTGGCTTCCTACT  
AATGTCACGTTTGGTTGTAAATACGGCAATCAGTTCCAACCGAAGGATGA

1351 AATAATACACAACCTTTTGTTACAACCATTGTTGGGCTATATGGTCTGTG  
TTATTATGTGTTGGAAAACAATGTTGGTAACAACCCGATATACCAGACAC

1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA  
GAACGTTTCGTTTATCACCTGTTTCATACCTATCTCCTGACATCGTCACTTT

1451 AGGCTGAACAACAGTGGGCTCTTTATGCAGATGGTTCAATACGTCCTCAG  
TCCGACTTGTGTGTCACCCGAGAAATACGTCTACCAAGTTATGCAGGAGTC

1501 CAAAACCGAGATAATTGCCTTACAAGTGATTCTAATATACGGGAAACAGT  
GTTTTGGCTCTATTAACGGAATGTTCACTAAGATTATATGCCCTTTGTCA

1551 TGTTAAGATCCTCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT  
ACAATTCTAGGAGAGAAACACCGGGACGTAGGAGACCGGTTGCTACCTACA

1601 TCAAGAATGATGGAACCATTTTAAATTTGTATAGTGGATTGGTGTTAGAT  
AGTTCTTACTACCTTGGTAAAATTTAAACATATCACCTAACCACAATCTA

1651 GTGAGGCGATCGGATCCGAGCCTTAAACAAATCATTCTTTACCCCTCTCCA  
CACTCCGCTAGCCTAGGCTCGGAATTTGTTTAGTAAGAAATGGGAGAGGT

1701 TGGTGACCCAAACCAAATATGGTTACCATTATTTTGATAGACAGATTACT  
ACCACTGGGTTTGGTTTATACCAATGGTAATAAACTATCTGTCTAATGA

1751 CTCTTGCAGTGTGTGTGTCCTGCCATGAAAATAGATGGCTTAAATAAAAA  
GAGAACGTACACACACAGGACGGTACTTTTATCTACCGAATTTATTTTT

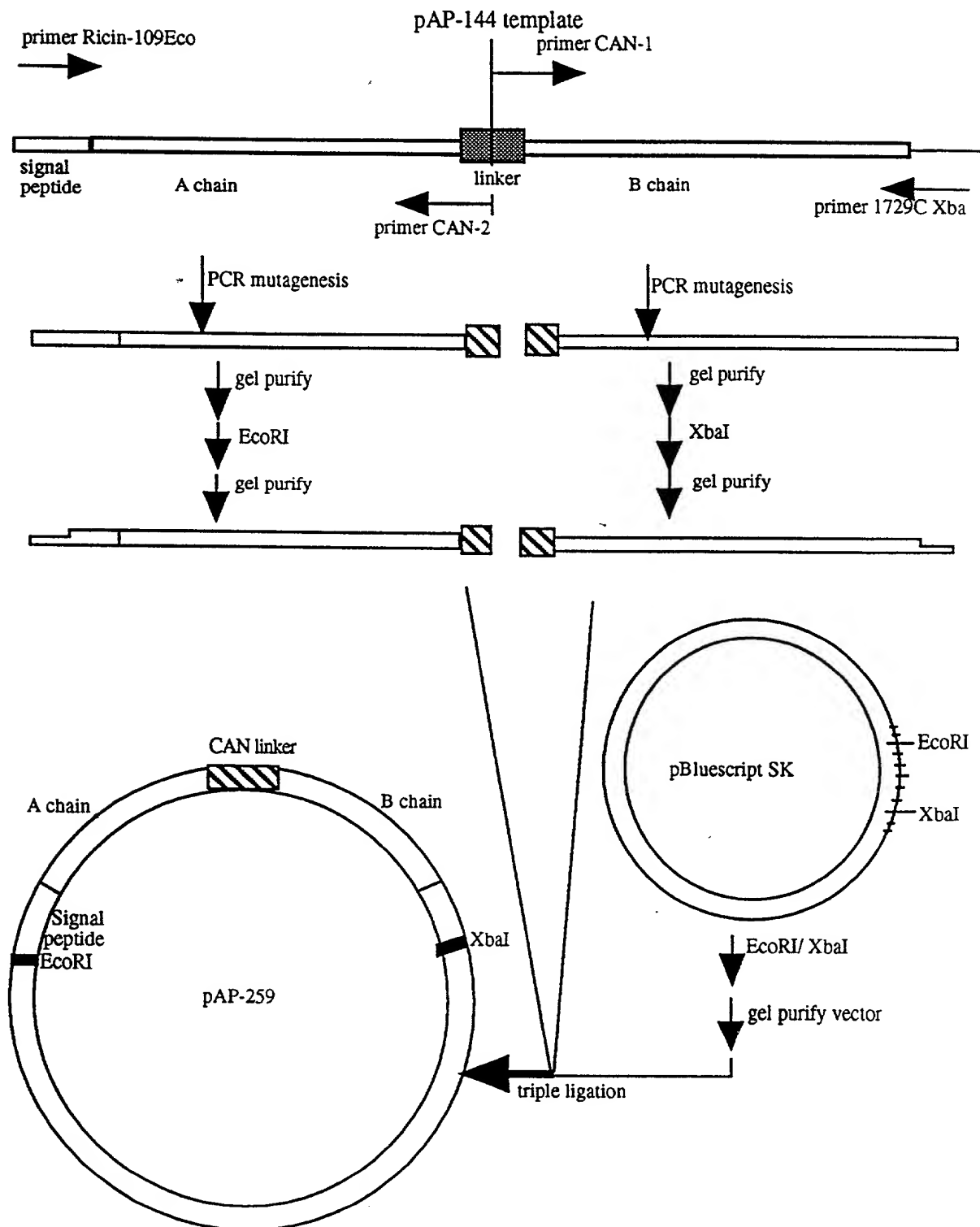
1801 GGACATTGTAAATTTTGTAACTGAAAGGACAGCAAGTTATATCGAATTCC  
CCTGTAACATTTAAACATTGACTTTCCTGTCGTTCAATATAGCTTAAGG

1851 TGCAG  
ACGTC

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**FIGURE 25A**



## FIGURE 25B

### WT preprorin linker

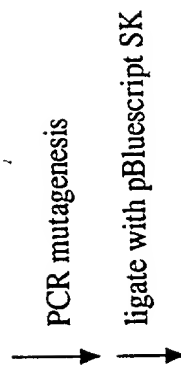
primer CAN-1

5' - TTCAGGCTAAATTTTAATGCTGAT -3'

TCCTTGCTTATAAGGCCAGTGGTGCCAAATTTTAAT  
AGAAACGAATATTCCTGGTACCCACGGTTAAAAATTA

3' - AGCAGTGTCAAAAGATTCCGGACGTTTCAAG-5'

primer CAN-2



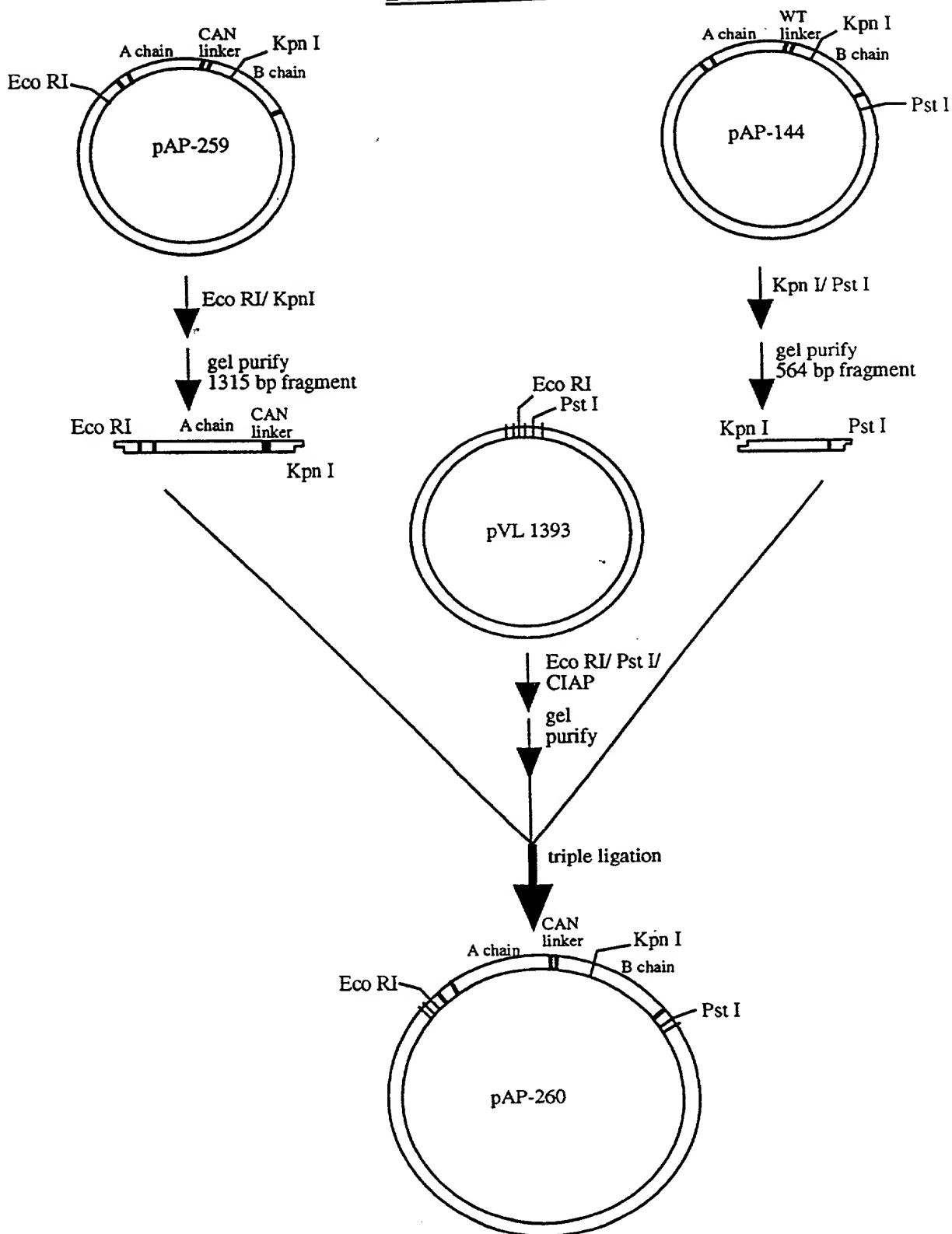
### pAP 259 linker (CAN variant)

TCTAAGCCTGC AAAAGTTCTTCAGGCTAAATTTTAAT  
AGATTCCGACGTTTCAAGAAGTCCGATTAAAAATTA



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**FIGURE 25C**



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## FIGURE 25D

10 20 30 40 50

1 GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT  
CTTAAGTACTTTGGCCCTCCTTTATGATAACATTATACCTACATACGTCA

51 GGCAACATGGCTTTGTTTGGATCCACCTCAGGGTGGTCTTTCACATTAG  
CCGTTGTACCGAAACAAACCTAGGTGGAGTCCCACCAGAAAGTGTAATC

101 AGGATAACAACATATTCCCCAAACAATACCCAATTATAAACTTTACCACA  
TCCTATTGTTGTATAAGGGGTTTGTATGGGTAAATATTTGAAATGGTGT

151 GCGGGTGCCACTGTGCAAAGCTACACAACTTTATCAGAGCTGTTTCGCGG  
CGCCACGGTGACACGTTTCGATGTGTTGAAATAGTCTCGACAAGCGCC

201 TCGTTTAACAACCTGGAGCTGATGTGAGACATGATATACCAGTGTGCCAA  
AGCAAATTGTTGACCTCGACTACACTCTGTACTATATGGTCACAACGGTT

251 ACAGAGTTGGTTTGCCTATAAACCAACGGTTTATTTTAGTTGAACTCTCA  
TGTCTCAACCAACGGATATTGTTGTTGCCAAATAAAATCAACTTGAGAGT

301 AATCATGCAGAGCTTTCGTACATTAGCGCTGGATGTCACCAATGCATA  
TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT

351 TGTGGTCGGCTACCGTGCTGGAAATAGCGCATATTTCTTTTCATCCTGACA  
ACACCAGCCGATGGCAGCACCTTTATCGCGTATAAAGAAAGTAGGACTGT

401 ATCAGGAAGATGCAGAAGCAATCACTCATCTTTTCACTGATGTTCAAAAT  
TAGTCCTTCTACGTCTTCGTTAGTGAGTAGAAAAGTGACTACAAGTTTTA

451 CGATATACATTTCGCTTTGGTGGTAATTATGATAGACTTGAACAACTTGC  
GCTATATGTAAGCGGAAACCACCATTAATACTATCTGAACTTGTGTAACG

501 TGGTAATCTGAGAGAAAATATCGAGTTGGGAAATGGTCCACTAGAGGAGG  
ACCATTAGACTCTCTTTTATAGCTCAACCCTTTACCAGGTGATCTCCTCC

551 CTATCTCAGCGCTTTATTATTACAGTACTGGTGGCACTCAGCTTCCAAC  
GATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA

601 CTGGCTCGTTTCCTTTATAATTTGCATCCAAATGATTTTCAAGCAGCAAG  
GACCGAGCAAGGAAATATTAAACGTAGGTTTACTAAAGTCTTCGTCGTTT

651 ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA  
TAAGGTTATATAACTCCCTCTTTACGCGTGCTCTTAATCCATGTTGGCCT

701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGGAGA  
CTAGACGTGGTCTAGGATCGCATTAAATGTGAACCTTTATCAACCCCTCT

751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTTGCTAGTCCAAT  
GAAAGGTGACGTTAAGTTCTCAGATTGGTTCCCTCGGAAACGATCAGGTTA

801 TCAACTGCAAAGACGTAATGGTTCCAAATTCAGTGTGTACGATGTGAGTA  
AGTTGACGTTTCTGCATTACCAAGGTTTAAAGTCACACATGCTACACTCAT

851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGCACCTCCACCA  
ATAATTAGGGATAGTATCGAGAGTACCACATATCTACGCGTGGAGGTGGT

901 TCGTCACAGTTTCTAAGCCTGCAAAGTTCTTCAGGCTAAATTTTAATGC  
AGCAGTGTCAAAAGATTTCGGACGTTTCAAGAAGTCCGATTTAAATACG

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**FIGURE 25D (CONT'D)**

951 TGATGTTTGTATGGATCCTGAGCCCATAGTGCCTATCGTAGGTCGAAATG  
ACTACAAACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTTAC

1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA  
CAGATACACAACATAAATCCCTACCTTCTAAGGTGTTGCCTTTGCGTTAT

1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT  
GTCAACACCGGTACGTTTCAGATTATGTCTACGTTTTCAGTCGAGACCTGAAA

1101 GAAAAGAGACAATACTATTTCGATCTAATGGAAAGTGTTTAACTACTTACG  
CTTTTCTCTGTTATGATAAGCTAGATTACCTTTTCAAAATTGATGAATGC

1151 GGTCAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA  
CCATGTTCAGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT

1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC  
TGACTACGGTGGGCGACCGTTTATACCCTATTACCTTGGTAGTATTTAGG

1251 CAGATCTAGTCTAGTTTTAGCAGCGACATCAGGGAACAGTGGTACCACAC  
GTCTAGATCAGATCAAAATCGTCGCTGTAGTCCCTTGTCACCATGGTGTG

1301 TTACAGTGCAAACCAACATTTATGCCGTTAGTCAAGGTTGGCTTCCTACT  
AATGTCACGTTTGGTTGTAAATACGGCAATCAGTTCCAACCGAAGGATGA

1351 AATAATACACAACCTTTTGTACAAACCATTTGTTGGGCTATATGGTCTGTG  
TTATTATGTGTTGGAAAACAATGTTGGTAACAACCCGATATACCAGACAC

1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAG  
GAACGTTTCGTTTATCACCTGTTTCATACCTATCTCCTGACATCGTCACTTT

1451 AGGCTGAACAACAGTGGGCTCTTTATGCAGATGGTTCAATACGTCCTCAG  
TCCGACTTGTGTACCCGAGAAATACGTCTACCAAGTTATGCAGGAGTC

1501 CAAAACCGAGATAATTGCCTTACAAGTGATTCTAATATACGGGAAACAGT  
GTTTTGGCTCTATTAAACGAATGTTCACTAAGATTATATGCCCTTTGTCA

1551 TGTTAAGATCCTCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT  
ACAATTCTAGGAGAGAACACCGGGACGTAGGAGACCGGTTGCTACCTACA

1601 TCAAGAATGATGGAACCATTTTAAATTTGTATAGTGGATTGGTGTAGAT  
AGTTCTTACTACCTTGGTAAAAATTTAAACATATCACCTAACCAATCTA

1651 GTGAGGCGATCGGATCCGAGCCTTAAACAAATCATTCTTTACCCCTCTCCA  
CACTCCGCTAGCCTAGGCTCGGAATTGTTTGTAGTAAGAAATGGGAGAGGT

1701 TGGTGACCCAAACCAAATATGGTTACCATTTATTTGATAGACAGATTACT  
ACCACTGGGTTTGGTTTATACCAATGGTAATAAACTATCTGTCTAATGA

1751 CTCTTGACGTGTGTGTGTCCTGCCATGAAAATAGATGGCTTAAATAAAAA  
GAGAACGTACACACACAGGACGGTACTTTTATCTACCGAATTTATTTTT

1801 GGACATTGTAAATTTTGTAACTGAAAGGACAGCAAGTTATATCGAATTCC  
CCTGTAACATTTAAACATTGACTTTCTGTGCTCAATATAGCTTAAGG

1851 TGCAG  
ACGTC

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**FIGURE 26**

Ricin linker (wild type):

A chain- S L L I R P V V P N F N -B chain

pAP-223/224 linker (MAL-A):

A chain- Q V V Q L Q N Y D E E D -B chain

pAP-225/226 linker (MAL-B):

A chain- L P I F G E S E D N D E -B chain

pAP-227/228 linker (MAL-C):

A chain- Q V V T G E A I S V T M -B chain

pAP-229/230 linker (MAL-D):

A chain- A L E R T F L S F P T N -B chain

pAP-231/pAP-232 linker (MAL-E):

A chain- K F Q D M L N I S Q H Q -B chain

A chain- S L L I R P V V P N F N -B chain

A chain- S G V V N A S C R L A N -B chain

A chain- S S Y V K A S V S P E N -B chain

A chain- S A L V N A S S A H V N -B chain

A chain- S T Y L Q-A S E K F K N -B chain

A chain- S S I L N A S V P N F N -B chain

A chain- S Q D V N A V E A S S N -B chain

A chain- S V Y L Q A S T G Y G N -B chain

A chain- S K Y L Q A N E V I T N -B chain

A chain- S E L R T Q S F S N W N -B chain

A chain- S E L W S Q G I D D D N -B chain

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**FIGURE 28**

Ricin linker (wild type):

A chain- S L L I R P V V P N F N -B chain

pAP-259/260 linker (CAP-A):

A chain- S K P A K F F R L N F N -B chain

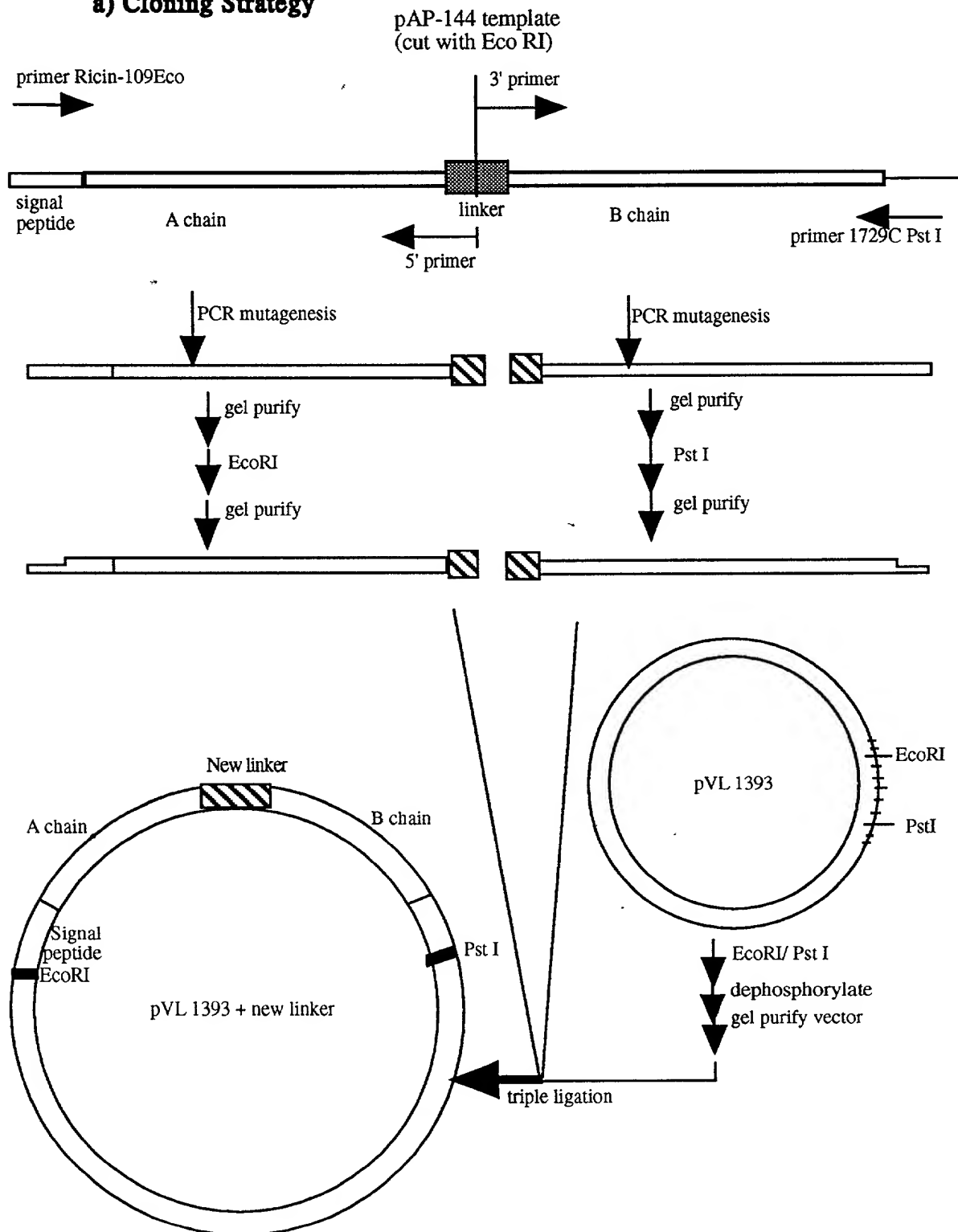
pAP-261/262 linker (CAP-B):

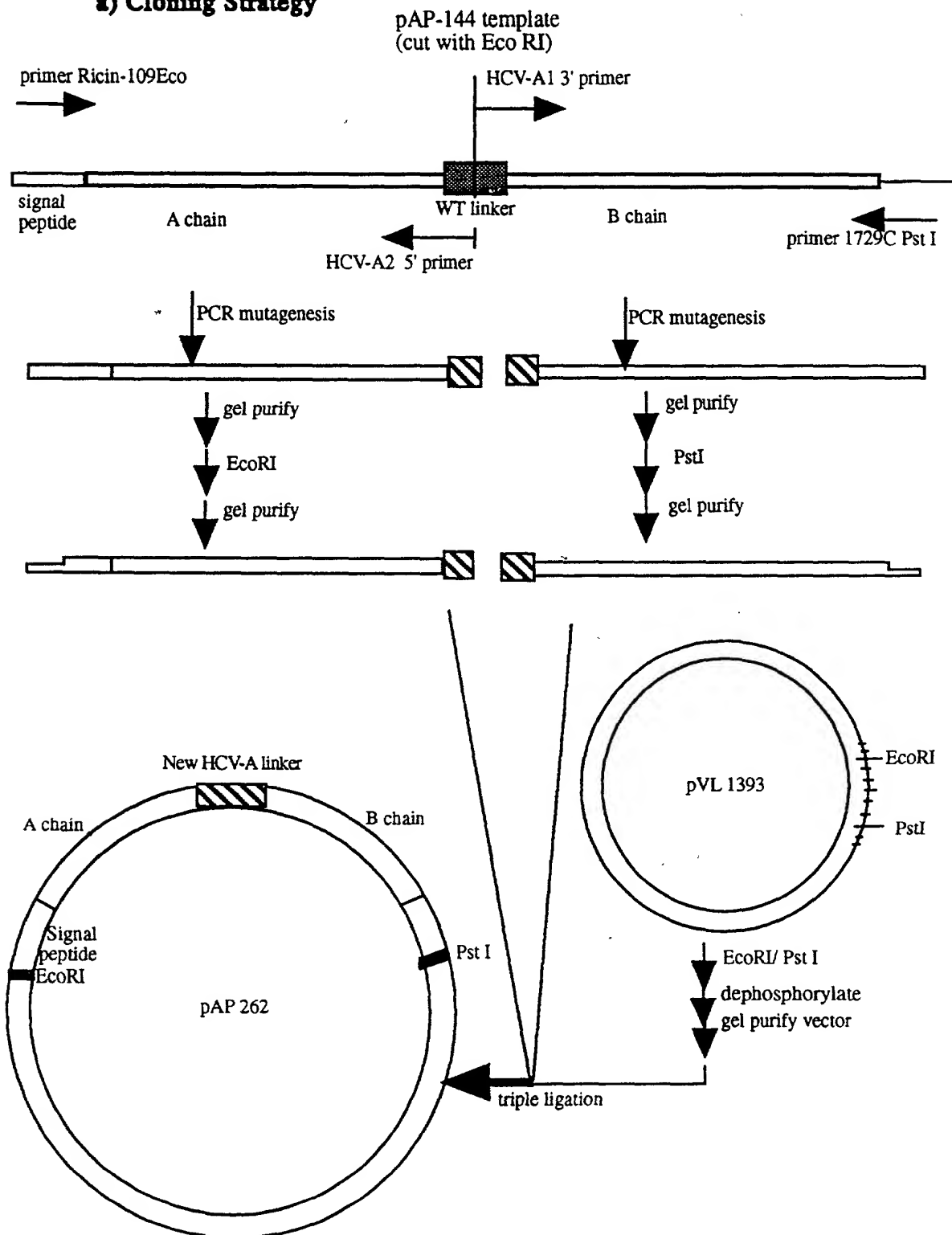
A chain- S K P I E F F R L N F N -B chain

pAP-263/264 linker (CAP-C):

A chain- S K P A E F F A L N F N -B chain

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**FIGURE 29****PCR Mutagenesis of Preproricin Gene to Create A Variant Gene in Baculovirus Transfer Vector, pVL 1393****a) Cloning Strategy**

**FIGURE 30A****PCR Mutagenesis of Preprorincin Gene to Create An HCV-A Variant Gene in Baculovirus Transfer Vector, pVL 1393****a) Cloning Strategy**



# FIGURE 30B

## Sequence of HCV-A Linker Region

### WT preprorin linker

primer HCV-A1

5' - TCGACATGGGTTTAAATGCTGATGTT -3'  
 TCTTTGCTTATAAGGCCAGTGGTGCCAAATTTAAT  
 AGAAACGAATATCCGGTCACCCAGGTTTAAATA  
 3' - GGTAGCAGTGTCAAACTAACCTCCATCACTGT 5'

5' primer HCV-A2

PCR mutagenesis

ligate with pVL1393

pAP 262 linker  
 (HCV-A variant)

GATTGGAGGTAGTGACATCGACATGGGTTTAAAT  
 CTAAACCTCCATCACTGTAGCTGTACCCAAAAATA

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## FIGURE 30C (P1)

Sequence of pAP262 insert

10 20 30 40 50  
| | | | |  
1 GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT  
CTTAAGTACTTTGGCCCTCCTTTATGATAACATTATACCTACATACGTCA  
51 GGCAACATGGCTTTGTTTGGATCCACCTCAGGGTGGTCTTTCACATTAG  
CCGTTGTACCGAAACAAAACCTAGGTGGAGTCCCACCAGAAAGTGTATC  
101 AGGATAACAACATATCCCCAAACAATACCCAATTATAAACTTTACCACA  
TCCTATTGTTGTATAAGGGGTTTGTATGGGTTAATATTTGAAATGGTGT  
151 GCGGGTGCCACTGTGCAAAGCTACACAACTTTATCAGAGCTGTTGCGGG  
CGCCCACGGTGACACGTTTCGATGTGTTTGAAATAGTCTCGACAAGCGCC  
201 TCGTTTAACAACCTGGAGCTGATGTGAGACATGATATAACAGTGTGCCAA  
AGCAAATTGTTGACCTCGACTACACTCTGTACTATATGGTCACAACGGTT  
251 ACAGAGTTGGTTTGCCTATAAACCAACGGTTTATTTTAGTTGAACTCTCA  
TGTCTCAACCAAACGGATATTTGGTTGCCAAATAAAATCAACTTGAGAGT  
301 AATCATGCAGAGCTTTCTGTTACATTAGCGCTGGATGTCACCAATGCATA  
TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT  
351 TGTGGTCGGCTACCGTGCTGGAAATAGCGCATATTTCTTTTCATCCTGACA  
ACACCAGCCGATGGCACGACCTTTATCGCGTATAAAGAAAGTAGGACTGT  
401 ATCAGGAAGATGCAGAAGCAATCACTCATCTTTTCACTGATGTTCAAAT  
TAGTCCTTCTACGTCTTCGTTAGTGAGTAGAAAAGTGACTACAAGTTTTA  
451 CGATATACATTGCGCTTTGGTGGTAATTATGATAGACTTGAACAACCTTGC  
GCTATATGTAAGCGGAAACCACCATTAATACTATCTGAACTTGTGTAACG  
501 TGGTAATCTGAGAGAAAATATCGAGTTGGGAAATGGTCCACTAGAGGAGG  
ACCATTAGACTCTCTTTTATAGCTCAACCCTTTACCAGGTGATCTCCTCC  
551 CTATCTCAGCGCTTTATTATTACAGTACTGGTGGCACTCAGCTTCCAAC  
GATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA  
601 CTGGCTCGTTCCTTTATAATTTGCATCCAAATGATTTCAGAAGCAGCAAG  
GACCGAGCAAGGAAATATTAACGTAGGTTTACTAAAGTCTTCGTGCTTC  
651 ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA  
TAAGGTTATATAACTCCCTCTTTACGCGTGCTCTTAATCCATGTTGGCCT  
701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGGAGA  
CTAGACGTGGTCTAGGATCGCATTAAATGTGAACTCTTATCAACCCCCCTCT  
751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTTGCTAGTCCAAT  
GAAAGGTGACGTTAAGTTCTCAGATTGGTTTCCTCGGAAACGATCAGGTTA

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**FIGURE 30C (P2)**

801 TCAACTGCAAAGACGTAATGGTTCCAAATTCAGTGTGTACGATGTGAGTA  
AGTTGACGTTTCTGCATTACCAAGGTTTAAGTCACACATGCTACACTCAT

851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGCACCTCCACCA  
ATAATTAGGGATAGTATCGAGAGTACCACATATCTACGCGTGGAGGTGGT

901 TCGTCACAGTTTGATTTGGAGGTAGTGACATCGACATGGGTTTTTAATGC  
AGCAGTGTCAAACCTAAACCTCCATCACTGTAGCTGTACCCAAAAATTACG

951 TGATGTTTGTATGGATCCTGAGCCCATAGTGCGTATCGTAGGTCGAAATG  
ACTACAAACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTTAC

1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA  
CAGATACACAACCTACAATCCCTACCTTCTAAGGTGTTGCCTTTGCGTTAT

1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT  
GTCAACACCGGTACGTTTACGATTATGTCTACGTTTAGTCGAGACCTGAAA

1101 GAAAAGAGACAATACTATTTCGATCTAATGGAAAGTGTTTAACTACTTACG  
CTTTTCTCTGTTATGATAAGCTAGATTACCTTTCACAAATTGATGAATGC

1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA  
CCATGTGAGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT

1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC  
TGACTACGGTGGGCGACCGTTTATACCTATTACCTTGGTAGTATTTAGG

1251 CAGATCTAGTCTAGTTTTAGCAGCGACATCAGGGAACAGTGGTACCACAC  
GTCTAGATCAGATCAAATCGTCGCTGTAGTCCCTTGTCAACCATGGTGTG

1301 TTACAGTGCAAACCAACATTTATGCCGTTAGTCAAGGTTGGCTTCCTACT  
AATGTCACGTTTGGTGTGTAATACGGCAATCAGTTCCAACCGAAGGATGA

1351 AATAATACACAACCTTTTGTTACAACCATTGTTGGGCTATATGGTCTGTG  
TTATTATGTGTTGGAAAACAATGTTGGTAACAACCCGATATACCAGACAC

1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA  
GAACGTTTCGTTTATCACCTGTTTACATACCTATCTCCTGACATCGTCACTTT

1451 AGGCTGAACAACAGTGGGCTCTTTATGCAGATGGTTCAATACGTCCTCAG  
TCCGACTTGTTGTCACCCGAGAAATACGTCTACCAAGTTATGCAGGAGTC

1501 CAAAACCGAGATAATTGCCTTACAAGTGATTCTAATATACGGGAAACAGT  
GTTTTGGCTCTATTAACGGAATGTTCACTAAGATTATATGCCCTTTGTCA

1551 TGTTAAGATCCTCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT  
ACAATTCTAGGAGAGAACACCGGGACGTAGGAGACCGGTTGCTACCTACA

1601 TCAAGAATGATGGAACCATTTTAAATTTGTATAGTGGATTGGTGTAGAT  
AGTTCTTACTACCTTGGTAAAATTTAAACATATCACCTAACCACAATCTA

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**FIGURE 30C (P3)**

1651 GTGAGGCGATCGGATCCGAGCCTTAAACAAATCATTCTTTACCCTCTCCA  
CACTCCGCTAGCCTAGGCTCGGAATTTGTTTAGTAAGAAATGGGAGAGGT

1701 TGGTGACCCAAACCAAATATGGTTACCATTATTTTGATAGACAGATTACT  
ACCACTGGGTTTGGTTTATACCAATGGTAATAAACTATCTGTCTAATGA

1751 CTCTTGCAAGTGTGTGTGTCCTGCCATGAAAATAGATGGCTTAAATAAAAA  
GAGAACGTCACACACACAGGACGGTACTTTTATCTACCGAATTTATTTTT

1801 GGACATTGTAAATTTTGTAACTGAAAGGACAGCAAGTTATATCGAATTCC  
CCTGTAACATTTAAACATTGACTTTCCTGTCGTTCAATATAGCTTAAGG

1851 TGCAG  
ACGTC

Total number of bases is: 1855.

Sequence name: PAP262

00440 "PAP262"

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**FIGURE 30D**

**-Amino Acid Sequence Comparison of Mutant  
Preproricin Linker Region of HCV-A to Wild Type**

Wild type Ricin linker: A chain- S L L I R P V V P N F N -B chain

pAP-262 linker: A chain- D L E V V T S T W V F N -B chain  
(HCV-A linker)

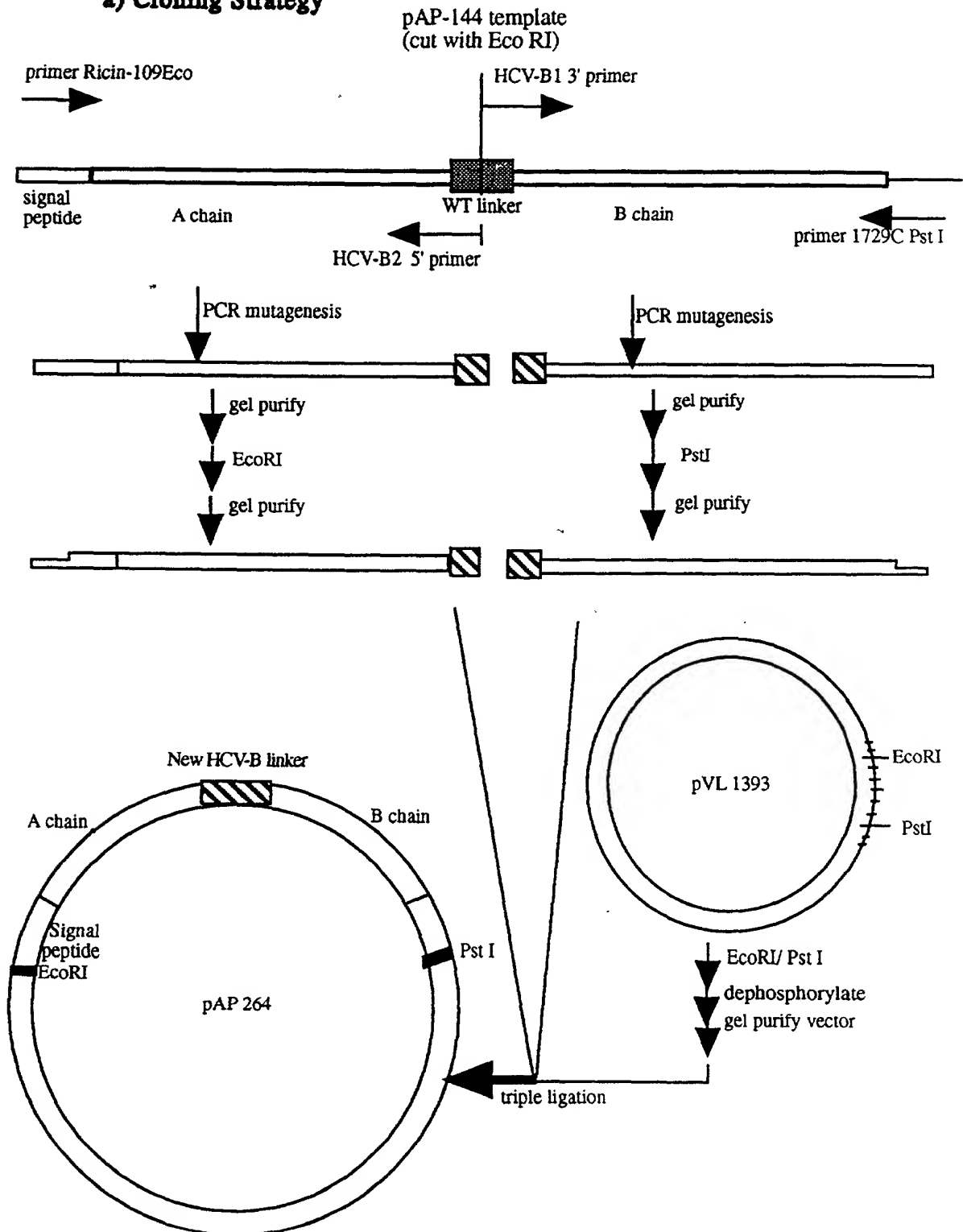
00440 151 041400

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# FIGURE 31A

**PCR Mutagenesis of Preprorizin Gene to Create An HCV-B Variant Gene in Baculovirus Transfer Vector, pVL 1393**

## a) Cloning Strategy



# **FIGURE 31B**

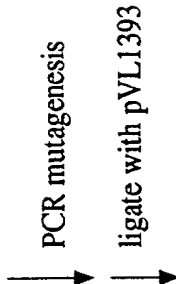
## **Sequence of HCV-B Linker Region**

### **WT preprorin linker**

primer HCV-B1

5' - GCGTCACACCCCTTTTAAATGCTGATGTT -3'  
 \* \* \* \* \*  
 TCTTTGCTTATAAGGCCAGTGGTGCCAAATTTAAT  
 AGAAACGAATATTCCGGTCACCCAGGTTAAATTA  
 \* \* \* \* \*  
 3' - GGTAGCAGTGTCAAACTACTACCTTCTCACA-5'

5' primer HCV-B2



pAP 264 linker  
 (HCV-B variant)

GATGAGATGGAAGAGTGGTGCCACACCTTTTAAAT  
 CTACTCTACCTTCTCACACCGCAGTGGGAAAAATTA

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## FIGURE 31C (P1)

Sequence of pAP264 insert

```

      10      20      30      40      50
      |      |      |      |      |
1  GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT
   CTTAAGTACTTTGGCCCTCCTTTATGATAACATTATACCTACATACGTCA

51  GGCAACATGGCTTTGTTTTGGATCCACCTCAGGGTGGTCTTTCACATTAG
   CCGTTGTACCGAAACAAAACCTAGGTGGAGTCCCACCAGAAAGTGTAATC

101 AGGATAACAACATATTCCCCAAACAATACCCAATTATAAACTTTACCACA
   TCCTATTGTTGTATAAGGGGTTTGTTATGGGTAAATATTTGAAATGGTGT

151 GCGGGTGCCACTGTGCAAAGCTACACAACTTTATCAGAGCTGTTGCGGG
   CGCCACGGTGACACGTTTCGATGTGTTTGAAATAGTCTCGACAAGCGCC

201 TCGTTTAAACAACTGGAGCTGATGTGAGACATGATATACCAAGTGTGCCAA
   AGCAAATTGTTGACCTCGACTACACTCTGTACTATATGGTCACAACGGTT

251 ACAGAGTTGGTTTGCCTATAAACCAACGGTTTATTTTAGTTGAACTCTCA
   TGTCTCAACCAACGGATATTTGGTTGCCAAATAAAATCAACTTGAGAGT

301 AATCATGCAGAGCTTTCTGTTACATTAGCGCTGGATGTCACCAATGCATA
   TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT

351 TGTGGTCGGCTACCGTGCTGGAAATAGCGCATATTTCTTTCATCCTGACA
   ACACCAGCCGATGGCACGACCTTTATCGCGTATAAAGAAAGTAGGACTGT

401 ATCAGGAAGATGCAGAAGCAATCACTCATCTTTTCACTGATGTTCAAAAT
   TAGTCCTTCTACGTCTTCGTTAGTGAGTAGAAAAGTGACTACAAGTTTTA

451 CGATATACATTTCGCCTTTGGTGGTAATTATGATAGACTTGAACAACTTGC
   GCTATATGTAAGCGGAAACCACCATTAATACTATCTGAAGTTGTTGAACG

501 TGGTAATCTGAGAGAAAATATCGAGTTGGGAAATGGTCCACTAGAGGAGG
   ACCATTAGACTCTCTTTTATAGCTCAACCCTTTACCAGGTGATCTCCTCC

551 CTATCTCAGCGCTTTATTATTACAGTACTGGTGGCACTCAGCTTCCAAC
   TATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA

601 CTGGCTCGTTCCTTTATAATTTGCATCCAAATGATTTCAGAAGCAGCAAG
   GACCGAGCAAGGAAATATTAAACGTAGGTTTACTAAAGTCTTCGTCGTT

651 ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA
   TAAGGTTATATAACTCCCTCTTTACGCGTGCTCTTAATCCATGTTGGCCT

701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGGAGA
   CTAGACGTGGTCTAGGATCGCATTAATGTGAACTCTTATCAACCCCTCT

751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTTGCTAGTCCAAT
   GAAAGGTGACGTTAAGTTCTCAGATTGGTTCCTCGGAAACGATCAGGTTA
```

05440156



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**FIGURE 31C (P2)**

801 TCAACTGCAAAGACGTAATGGTTCCAAATTCAGTGTGTACGATGTGAGTA  
AGTTGACGTTTCTGCATTACCAAGGTTTAAAGTCACACATGCTACACTCAT

851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGCACCTCCACCA  
ATAATTAGGGATAGTATCGAGAGTACCACATATCTACGCGTGGAGGTGGT

901 TCGTCACAGTTTGATGAGATGGAAGAGTGTGCGTCACACCTTTTTAATGC  
AGCAGTGTCAAACACTCTACCTTCTCACACGCAGTGTGGAAAAATTACG

951 TGATGTTTGTATGGATCCTGAGCCCATAGTGCATCGTAGGTCGAAATG  
ACTACAAACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTTAC

1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA  
CAGATACACAACACTACAATCCCTACCTTCTAAGGTGTTGCCTTTGCGTTAT

1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT  
GTCAACACCGGTACGTTTACAGATTATGTCTACGTTTAGTCGAGACCTGAAA

1101 GAAAAGAGACAATACTATTCGATCTAATGGAAAGTGTTTAACTACTTACG  
CTTTTCTCTGTTATGATAAGCTAGATTACCTTTCACAAATTGATGAATGC

1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA  
CCATGTCAGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT

1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC  
TGACTACGGTGGGCGACCGTTTATACCCTATTACCTTGGTAGTATTTAGG

1251 CAGATCTAGTCTAGTTTTAGCAGCGACATCAGGGAACAGTGGTACCACAC  
GTCTAGATCAGATCAAAATCGTCGCTGTAGTCCCTTGTCACCATGGTGTG

1301 TTACAGTGCAAACCAACATTTATGCCGTTAGTCAAGGTTGGCTTCCTACT  
AATGTCACGTTTGGTTGTAAATACGGCAATCAGTTCCAACCGAAGGATGA

1351 AATAATACACAACCTTTTGTTACAACCATTTGTTGGGCTATATGGTCTGTG  
TTATTATGTGTTGGAAAACAATGTTGGTAACAACCCGATATACCAGACAC

1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA  
GAACGTTTCGTTTATCACCTGTTTCATACCTATCTCCTGACATCGTCACTTT

1451 AGGCTGAACAACAGTGGGCTCTTTATGCAGATGGTTCAATACGTCCTCAG  
TCCGACTTGTGTGTCACCCGAGAAATACGTCTACCAAGTTATGCAGGAGTC

1501 CAAAACCGAGATAATTGCCTTACAAGTGAATCTAATATACGGGAAACAGT  
GTTTTGGCTCTATTAACGGAATGTTCACTAAGATTATATGCCCTTTGTCA

1551 TGTTAAGATCCTCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT  
ACAATTCTAGGAGAGAACACCGGGACGTAGGAGACCGGTTGCTACCTACA

1601 TCAAGAATGATGGAACCATTTTAAATTTGTATAGTGGATTGGTGTTAGAT  
AGTTCTTACTACCTTGGTAAAATTTAAACATATCACCTAACCAACATCTA

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**FIGURE 31C (P3)**

1651 GTGAGGCGATCGGATCCGAGCCTTAAACAAATCATTCTTTACCCCTCTCCA  
CACTCCGCTAGCCTAGGCTCGGAATTTGTTTAGTAAGAAATGGGAGAGGT

1701 TGGTGACCCAAACCAAATATGGTTACCATTATTTTGATAGACAGATTACT  
ACCACTGGGTTTGGTTTATACCAATGGTAATAAACTATCTGTCTAATGA

1751 CTCTTGCA GTGTGTGTCTCCTGCCATGAAAATAGATGGCTTAAATAAAAA  
GAGAACGTCACACACACAGGACGGTACTTTTATCTACCGAATTTATTTTT

1801 GGACATTGTAAATTTTGTA ACTGAAAGGACAGCAAGTTATATCGAATTCC  
CCTGTAACATTTAAACATTGACTTTCCTGTCGTTCAATATAGCTTAAGG

1851 TGCAG  
ACGTC

Total number of bases is: 1855.

Sequence name: PAP264

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**FIGURE 31D**

**-Amino Acid Sequence Comparison of Mutant  
Preproricin Linker Region of HCV-B to Wild Type**

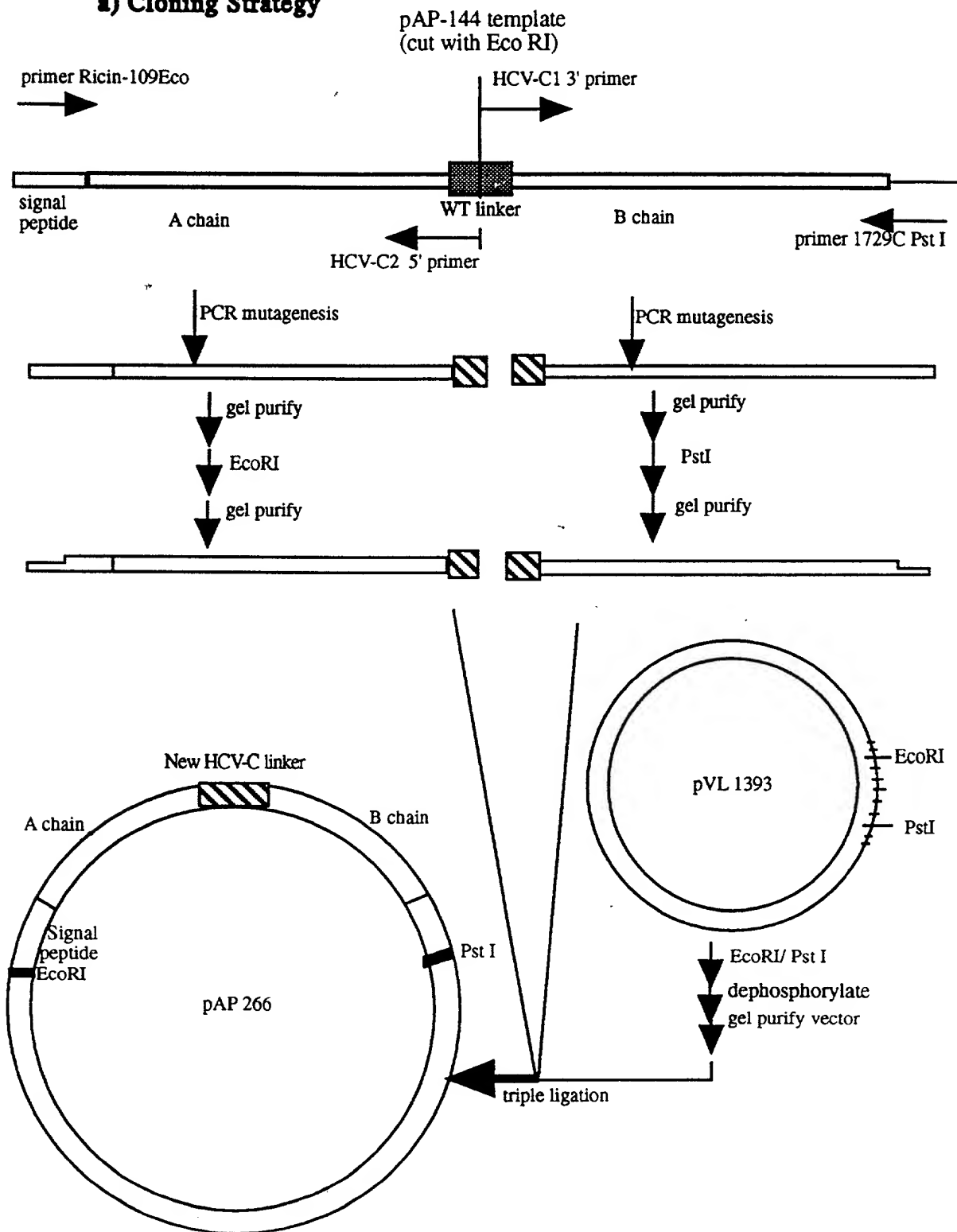
Wild type Ricin linker: A chain- S L L I R P V V P N F N -B chain

pAP-264 linker: A chain- D E M E E C A S H L F N -B chain  
(HCV-B linker)

00440.04400  
0955151

**FIGURE 32A**

**- PCR Mutagenesis of Preproricin Gene to Create An HCV-C Variant Gene in Baculovirus Transfer Vector, pVL 1393**

**a) Cloning Strategy**

# FIGURE 32B

## Sequence of HCV-C Linker Region

### WT preprorin linker

primer HCV-C1

5' - TCGATGTCATATTTTAATGCTGATGTT -3'

TCCTTGCCTTATAAGGCCAGTGGTGCCAAATTTTAAT  
AGAAACGAATATTCCCGGTACCCACGGTTAAATTA  
\*\*\*\*\*  
3' - GGTAGCAGTGTCAAACTCCTGCAACATACAAACA -5'

5' primer HCV-C2

PCR mutagenesis

ligate with pVL1393

pAP 266 linker  
(HCV-C variant)

GAGGACGTTGTATGTTGTCGATGTCATATTTTAAT  
CTCCTGCAACATACAAACAGCTACAGTATAAAATTA

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## FIGURE 32C (P1)

Sequence of pAP266 insert

```

      10      20      30      40      50
      |      |      |      |      |
1  GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT
   CTTAAGTACTTTGGCCCTCCTTTATGATAACATTATACCTACATACGTCA

51  GGCAACATGGCTTTGTTTTGGATCCACCTCAGGGTGGTCTTTCACATTAG
   CCGTTGTACCGAAACAAACCTAGGTGGAGTCCCACCAGAAAGTGTAATC

101 AGGATAACAACATATTCCCCAAACAATACCCAATTATAAACTTTACCACA
   TCCTATTGTTGTATAAGGGGTTTGTATGGGTAAATATTTGAAATGGTGT

151 GCGGGTGCCACTGTGCAAAGCTACACAACTTTATCAGAGCTGTTGCGGG
   CGCCACGGTGACACGTTTCGATGTGTTTGAAATAGTCTCGACAAGCGCC

201 TCGTTTAACAACCTGGAGCTGATGTGAGACATGATATACCACTGTTGCCAA
   AGCAAATTGTTGACCTCGACTACACTCTGTACTATATGGTCACAACGGTT

251 ACAGAGTTGGTTTGCCTATAAACCAACGGTTTATTTTAGTTGAACTCTCA
   TGTCTCAACCAACGGATATTTGGTTGCCAAATAAAATCAACTTGAGAGT

301 AATCATGCAGAGCTTCTGTTACATTAGCGCTGGATGTCACCAATGCATA
   TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT

351 TGTGGTCTGGCTACCGTGCTGGAAATAGCGCATATTTCTTTCATCCTGACA
   ACACCAGCCGATGGCAGACCTTTATCGCGTATAAAGAAAGTAGGACTGT

401 ATCAGGAAGATGCAGAAGCAATCACTCATCTTTTCACTGATGTTCAAAT
   TAGTCTTCTACGTCTTCGTTAGTGAGTAGAAAAGTGACTACAAGTTTTA

451 CGATATACATTGCGCTTTGGTGGTAATTATGATAGACTTGAACAACCTTGC
   GCTATATGTAAGCGGAAACCACCATTAACTATCTGAACTTGTGTAACG

501 TGGTAATCTGAGAGAAATATCGAGTTGGGAAATGGTCCACTAGAGGAGG
   ACCATTAGACTCTCTTTTATAGCTCAACCCTTTACCAGGTGATCTCCTCC

551 CTATCTCAGCGCTTTATTATTACAGTACTGGTGGCACTCAGCTTCCAAC
   TATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA

601 CTGGCTCGTTCCTTTATAATTTGCATCCAAATGATTTCAGAAGCAGCAAG
   GACCGAGCAAGGAAATATTAACGTAGGTTTACTAAAGTCTTCGTCGTTT

651 ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA
   TAAGGTTATATAACTCCCTCTTACGCGTGCTCTTAATCCATGTTGGCCT

701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGGAGA
   CTAGACGTGGTCTAGGATCGCATTAAATGTGAACTCTTATCAACCCCTCT

751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTTGCTAGTCCAAT
   GAAAGGTGACGTTAAGTTCTCAGATTGGTTCCTCGGAAACGATCAGGTTA
```

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**FIGURE 32C (P2)**

801 TCAACTGCAAAGACGTAATGGTTCCAAATTCAGTGTGTACGATGTGAGTA  
AGTTGACGTTTCTGCATTACCAAGGTTTAAGTCACACATGCTACACTCAT

851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGCACCTCCACCA  
ATAATTAGGGATAGTATCGAGAGTACCACATATCTACGCGTGGAGGTGGT

901 TCGTCACAGTTTGAGGACGTTGTATGTTGTTTCGATGTCATATTTTAATGC  
AGCAGTGTCAAACCTCTGCAACATAACAAGCTACAGTATAAAATTACG

951 TGATGTTTGTATGGATCCTGAGCCCATAGTGCGTATCGTAGGTCGAAATG  
ACTACAAACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTTAC

1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA  
CAGATACACAACCTACAATCCCTACCTTCTAAGGTGTTGCCTTTGCGTTAT

1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT  
GTCAACACCGGTACGTTTACAGATTATGTCTACGTTTAGTCGAGACCTGAAA

1101 GAAAAGAGACAATACTATTTCGATCTAATGGAAAGTGTTTAACTACTTACG  
CTTTTCTCTGTTATGATAAGCTAGATTACCTTTCACAAATTGATGAATGC

1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA  
CCATGTCAGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT

1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC  
TGACTACGGTGGGCGACCGTTTATACCTATTACCTTGGTAGTATTTAGG

1251 CAGATCTAGTCTAGTTTTAGCAGCGACATCAGGGAACAGTGGTACCACAC  
GTCTAGATCAGATCAAATCGTCGCTGTAGTCCCTTGTCACCATGGTGTG

1301 TTACAGTGCAAACCAACATTTATGCCGTTAGTCAAGGTTGGCTTCCTACT  
AATGTCACGTTTGGTTGTAAATACGGCAATCAGTTCCAACCGAAGGATGA

1351 AATAATACACAACCTTTTGTACAAACCATTGTTGGGCTATATGGTCTGTG  
TTATTATGTGTTGGAAAACAATGTTGGTAACAACCCGATATACCAGACAC

1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA  
GAACGTTTCGTTTATCACCTGTTTCATACCTATCTCCTGACATCGTCACTTT

1451 AGGCTGAACAACAGTGGGCTCTTTATGCAGATGGTTCAATACGTCCTCAG  
TCCGACTTGTTGTCACCCGAGAAATACGTCTACCAAGTTATGCAGGAGTC

1501 CAAAACCGAGATAATTGCCTTACAAGTGATTCTAATATACGGGAAACAGT  
GTTTTGGCTCTATTAACGGAATGTTCACTAAGATTATATGCCCTTTGTCA

1551 TGTTAAGATCCTCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT  
ACAATTCTAGGAGAGAACACCGGGACGTAGGAGACCGGTTGCTACCTACA

1601 TCAAGAATGATGGAACCATTTTTAAATTTGTATAGTGGATTGGTGTAGAT  
AGTTCTTACTACCTTGGTAAAATTTAAACATATCACCTAACCACAATCTA

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**FIGURE 32C (P3)**

1651 GTGAGGCGATCGGATCCGAGCCTTAAACAAATCATTCTTTACCCTCTCCA  
CACTCCGCTAGCCTAGGCTCGGAATTGTTTAGTAAGAAATGGGAGAGGT

1701 TGGTGACCCAAACCAAATATGGTTACCATTATTTTGATAGACAGATTACT  
ACCACTGGGTTTGGTTTATACCAATGGTAATAAACTATCTGTCTAATGA

1751 CTCTTGCAGTGTGTGTGTCCTGCCATGAAAATAGATGGCTTAAATAAAAA  
GAGAACGTCACACACACAGGACGGTACTTTTATCTACCGAATTTATTTTT

1801 GGACATTGTAAATTTTGTAAGTAAAGGACAGCAAGTTATATCGAATTCC  
CCTGTAAACATTTAAACATTGACTTTCCTGTCGTTCAATATAGCTTAAGG

1851 TGCAG  
ACGTC

Total number of bases is: 1855.

Sequence name: PAP266

00440" 1515560



### -Amino Acid Sequence Comparison of Mutant Preproricin Linker Region of HCV-C to Wild Type

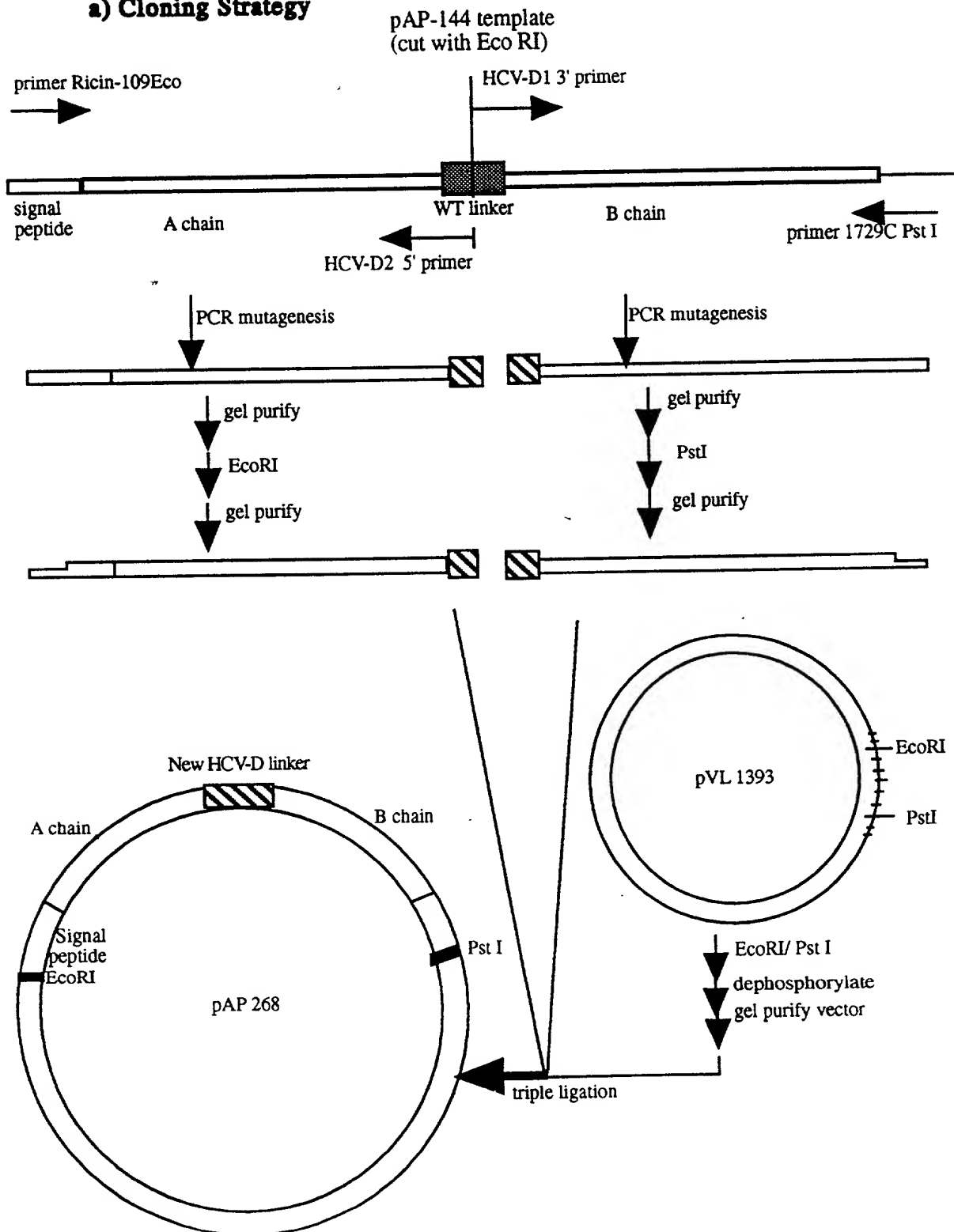
pAP-266 linker: A chain- E D V V C C S M S Y F N -B chain  
(HCV-C linker)

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# FIGURE 33A

**PCR Mutagenesis of Preproricin Gene to Create An HCV-D Variant Gene in Baculovirus Transfer Vector, pVL 1393**

## a) Cloning Strategy



# FIGURE 33B

## Sequence of HCV-D Linker Region

### WT preprorin linker

primer HCV-D1

5' - GCGCCAAATAACTGCTTATGCTGATGTTGTATG -3'

TCTTTGCTTATAAGGCCAGTGGTGCCAAATTTTAAT  
AGAAACGAATATTCGGTCCACCGTTTAAATTA

3' - GGTAGCAGTGTCAAATCCCCACCTCTAACGAT-5'

5' primer HCV-D2

PCR mutagenesis  
ligate with pVL1393

pAP 268 linker  
(HCV-D variant)

AAGGGTGGAGATTGCTAGCGCCAAATAACTGCTTAT  
TTCCCCACCTCTAACGATCGGGTTATTGACGAATA

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## FIGURE 33C (P1)

Sequence of pAP268 insert

```

      10      20      30      40      50
      |      |      |      |      |
1  GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT
   CTTAAGTACTTTGGCCCTCCTTTATGATAACATTATACCTACATACGTCA

51  GGCAACATGGCTTTGTTTTGGATCCACCTCAGGGTGGTCTTTCACATTAG
   CCGTTGTACCGAAACAAAACCTAGGTGGAGTCCCACCAGAAAGTGTAATC

101 AGGATAACAACATATTCCCCAAACAATACCCAATTATAAACTTTACCACA
   TCCTATTGTTGTATAAGGGGTTTGTATGGGTTAATATTTGAAATGGTGT

151 GCGGGTGCCACTGTGCAAAGCTACACAACTTTATCAGAGCTGTTTCGCGG
   CGCCCACGGTGACACGTTTCGATGTGTTTGAAATAGTCTCGACAAGCGCC

201 TCGTTTAACAACCTGGAGCTGATGTGAGACATGATATAACAGTGTTCGCAA
   AGCAAATTGTTGACCTCGACTACACTCTGTACTATATGGTCACAACGGTT

251 ACAGAGTTGGTTTGCCTATAAACCAACGGTTTATTTTAGTTGAACTCTCA
   TGTCTCAACCAAACGGATATTTGGTTGCCAAATAAAATCAACTTGAGAGT

301 AATCATGCAGAGCTTTCTGTTACATTAGCGCTGGATGTCACCAATGCATA
   TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT

351 TGTGGTCGGCTACCGTGCTGGAAATAGCGCATATTTCTTTCATCCTGACA
   ACACCAGCCGATGGCAGGACCTTTATCGCGTATAAAGAAAGTAGGACTGT

401 ATCAGGAAGATGCAGAAGCAATCACTCATCTTTTCACTGATGTTCAAAT
   TAGTCCTTCTACGTCTTCGTTAGTGAGTAGAAAAGTGACTACAAGTTTTA

451 CGATATACATTTCGCTTTTGGTGGTAATTATGATAGACTTGAACAACCTGC
   GCTATATGTAAGCGGAAACCACCATTAATACTATCTGAACTTGTTGAACG

501 TGGTAATCTGAGAGAAAATATCGAGTTGGGAAATGGTCCACTAGAGGAGG
   ACCATTAGACTCTCTTTTATAGCTCAACCCTTTACCAGGTGATCTCCTCC

551 CTATCTCAGCGCTTTATTATTACAGTACTGGTGGCACTCAGCTTCCAAC
   TATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA

601 CTGGCTCGTTCCTTTATAATTTGCATCCAAATGATTTTCAAGCAGCAAG
   GACCGAGCAAGGAAATATTAAACGTAGGTTTACTAAAGTCTTCGTCGTT

651 ATCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA
   TAAGGTTATATAACTCCCTCTTTACGCGTGCTCTTAATCCATGTTGGCCT

701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGGAGA
   CTAGACGTGGTCTAGGATCGCATTAATGTGAACTCTTATCAACCCCTCT

751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTTGCTAGTCCAAT
   GAAAGGTGACGTTAAGTTCTCAGATTGGTTCCTCGGAAACGATCAGGTTA
```

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**FIGURE 33C (P2)**

801 TCAACTGCAAAGACGTAATGGTTCCAAATTCAGTGTGTACGATGTGAGTA  
AGTTGACGTTTCTGCATTACCAAGGTTTAAGTCACACATGCTACACTCAT

851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGCACCTCCACCA  
ATAATTAGGGATAGTATCGAGAGTACCACATATCTACGCGTGGAGGTGGT

901 TCGTCACAGTTTAAAGGGGTGGAGATTGCTAGCGCCAATAACTGCTTATGC  
AGCAGTGTCAAATTCCTCCACCTCTAACGATCGCGGTATTGACGAATACG

951 TGATGTTTGTATGGATCCTGAGCCCATAGTGCCTATCGTAGGTCGAAATG  
ACTACAAACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTTAC

1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGAAACGCAATA  
CAGATACACAACCTACAATCCCTACCTTCTAAGGTGTTGCCTTTGCCTTAT

1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT  
GTCAACACCGGTACGTTTACAGATTATGTCTACGTTTAGTCGAGACCTGAAA

1101 GAAAAGAGACAATACTATTTCGATCTAATGGAAAGTGTTTAACTACTTACG  
CTTTTCTCTGTTATGATAAGCTAGATTACCTTTTCAAAATTGATGAATGC

1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA  
CCATGTCAGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT

1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC  
TGACTACGGTGGGCGACCGTTTATACCTATTACCTTGGTAGTATTTAGG

1251 CAGATCTAGTCTAGTTTTAGCAGCGACATCAGGGAACAGTGGTACCACAC  
GTCTAGATCAGATCAAAATCGTCGCTGTAGTCCCTTGTACCATTGGTGTG

1301 TTACAGTGCAAACCAACATTTATGCCGTTAGTCAAGGTTGGCTTCCTACT  
AATGTCACGTTTGGTTGTAAATACGGCAATCAGTTCCAACCGAAGGATGA

1351 AATAATACACAACCTTTTGTACAAACCATTGTTGGGCTATATGGTCTGTG  
TTATTATGTGTTGGAAAACAATGTTGGTAACAACCCGATATACCAGACAC

1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA  
GAACGTTTCGTTTATCACCTGTTTACCTATCTCCTGACATCGTCACTTT

1451 AGGCTGAACAACAGTGGGCTCTTTATGCAGATGGTTCAATACGTCCTCAG  
TCCGACTTGTTGTCACCCGAGAAATACGTCTACCAAGTTATGCAGGAGTC

1501 CAAAACCGAGATAATTGCCTTACAAGTGATTCTAATATACGGGAAACAGT  
GTTTTGGCTCTATTAACGGAATGTTCACTAAGATTATATGCCCTTTGTCA

1551 TGTTAAGATCCTCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT  
ACAATTCTAGGAGAGAACACCGGGACGTAGGAGACCGGTTGCTACCTACA

1601 TCAAGAATGATGGAACCATTTTAAATTTGTATAGTGGATTGGTGTTAGAT  
AGTTCTTACTACCTTGGTAAAATTTAAACATATCACCTAACCAACAATCTA

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**FIGURE 33C (P3)**

1651 GTGAGGCGATCGGATCCGAGCCTTAAACAAATCATTCTTTACCTCTCCA  
CACTCCGCTAGCCTAGGCTCGGAATTTGTTTAGTAAGAAATGGGAGAGGT

1701 TGGTGACCCAAACCAAATATGGTTACCATTATTTTGATAGACAGATTACT  
ACCACTGGGTTTGGTTTATACCAATGGTAATAAACTATCTGTCTAATGA

1751 CTCTTGCA GTGTGTGTGTCCTGCCATGAAAATAGATGGCTTAAATAAAAA  
GAGAACGTCACACACACAGGACGGTACTTTTATCTACCGAATTTATTTTT

1801 GGACATTGTAAATTTGTAAGTAAAGGACAGCAAGTTATATCGAATTCC  
CCTGTAACATTTAAACATTGACTTTCCTGTCTGTTCAATATAGCTTAAGG

1851 TGCAG  
ACGTC

Total number of bases is: 1855.

Sequence name: PAP268

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**FIGURE 33D**

**-Amino Acid Sequence Comparison of Mutant  
Preproricin Linker Region of HCV-D to Wild Type**

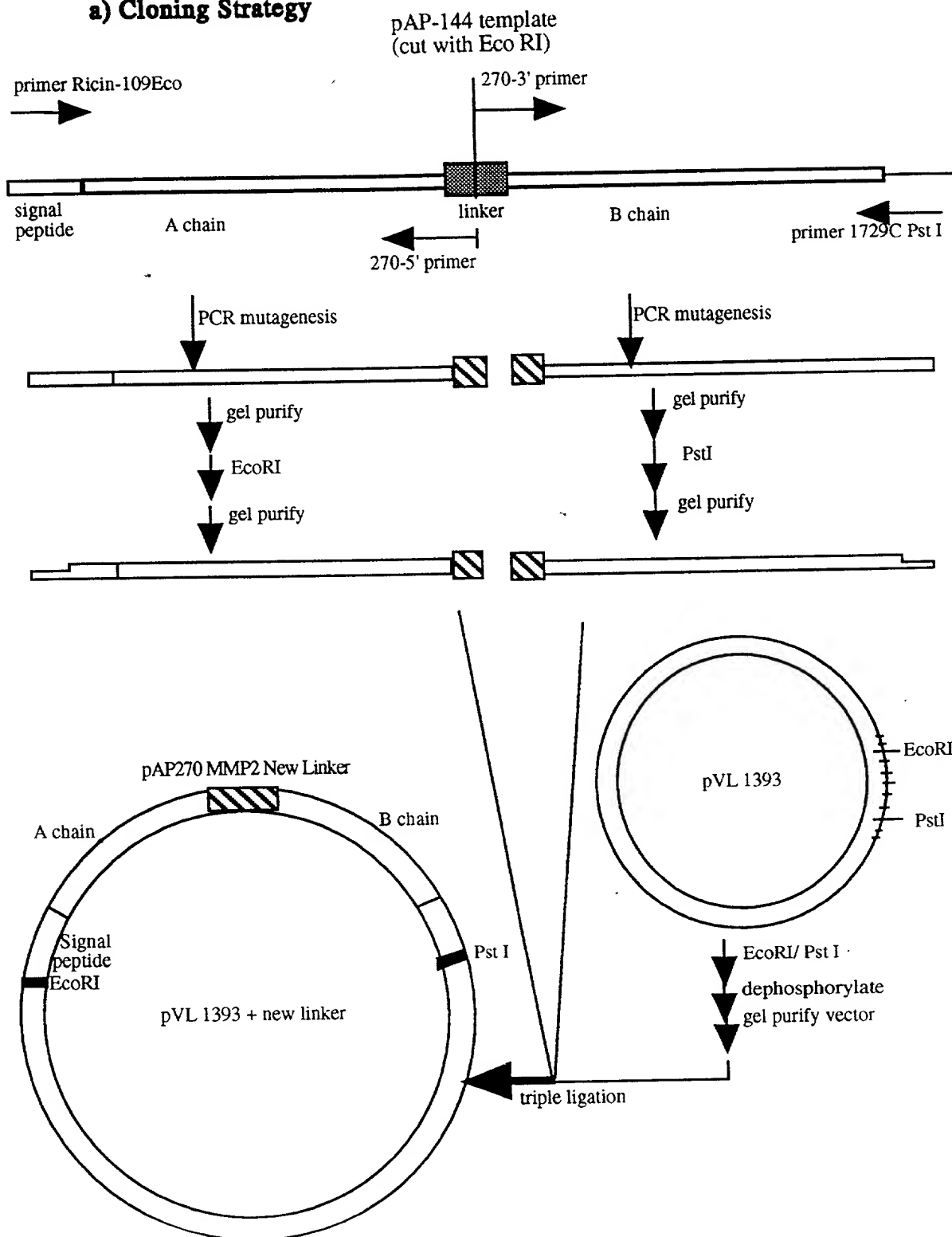
Wild type Ricin linker: A chain- S L L I R P V V P N F N -B chain

pAP-268 linker: A chain- K G W R L L A P I T A Y -B chain  
(HCV-D linker)

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**FIGURE 34A**

**PCR Mutagenesis of Preproricin Gene to Create A Variant Gene in Baculovirus Transfer Vector, pVL 1393**

**a) Cloning Strategy**



### Sequence of MMP-2 Linker Region

primer 270-3'

### 1) PCR mutagenesis

2) Ligate with pVL1393

**pAP 270 linker  
(MMP-2 variant)**

-----TCCTTGCCCCCTGGGTTTA|TGGGCTCCTAATTTTAAAT-----  
-----AGAAACGGGGACCCAAAT|ACCCGAGGATTAAAATTA-----

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**FIGURE 34C (P1)**

Sequence of pAP270 insert

```

      10      20      30      40      50
      |      |      |      |      |
1  GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT
   CTTAAGTACTTTGGCCCTCCTTTATGATAACATTATACCTACATACGTCA

51  GGCAACATGGCTTTGTTTTGGATCCACCTCAGGGTGGTCTTTCACATTAG
   CCGTTGTACCGAAACAAAACCTAGGTGGAGTCCCACCAGAAAGTGTAATC

101 AGGATAACAACATATTCCCCAAACAATACCCAATTATAAACTTTACCACA
   TCCTATTGTTGTATAAGGGGTTTGTATGGGTAAATATTTGAAATGGTGT

151 GCGGGTGCCACTGTGCAAAGCTACACAACTTTATCAGAGCTGTTGCGGG
   CGCCACGGTGACACGTTTCGATGTGTTTGAATAGTCTCGACAAGCGCC

201 TCGTTTAACAACCTGGAGCTGATGTGAGACATGATATAACAGTGTTGCCAA
   AGCAAATTGTTGACCTCGACTACACTCTGTACTATATGGTCACAACGGTT

251 ACAGAGTTGGTTTGCCTATAAACCAACGGTTTATTTTAGTTGAACTCTCA
   TGTCTCAACCAACGGATATTTGGTTGCCAAATAAAATCAACTTGAGAGT

301 AATCATGCAGAGCTTTCTGTTACATTAGCGCTGGATGTCACCAATGCATA
   TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT

351 TGTGGTCGGCTACCGTGCTGGAAATAGCGCATATTTCTTTCATCCTGACA
   ACACCAGCCGATGGCAGACCTTTATCGCGTATAAAGAAAGTAGGACTGT

401 ATCAGGAAGATGCAGAAGCAATCACTCATCTTTTCACTGATGTTCAAAAT
   TAGTCCTTCTACGTCTTCGTTAGTGAGTAGAAAAGTGACTACAAGTTTTA

451 CGATATACATTGCCTTTGGTGGTAATTATGATAGACTTGAACAACTTGC
   GCTATATGTAAGCGGAAACCACCATTAATACTATCTGAACTTGTTGAACG

501 TGGTAATCTGAGAGAAAATATCGAGTTGGGAAATGGTCCACTAGAGGAGG
   ACCATTAGACTCTCTTTTATAGCTCAACCCTTTACCAGGTGATCTCCTCC

551 CTATCTCAGCGCTTTATTATTACAGTACTGGTGGCACTCAGCTTCCAAC
   TATAGAGTCTCGGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA

601 CTGGCTCGTTCCTTTATAATTTGCATCCAAATGATTTCAGAAGCAGCAAG
   GACCGAGCAAGGAAATATTAAACGTAGGTTTACTAAAGTCTTCGTCGTTC

651 ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA
   TAAGGTTATATAACTCCCTCTTTACGCGTGCTCTTAATCCATGTTGGCCT

```

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**FIGURE 34C (P2)**

701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGGAGA  
CTAGACGTGGTCTAGGATCGCATTAAATGTGAACTCTTATCAACCCCCTCT

751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTTGCTAGTCCAAT  
GAAAGGTGACGTTAAGTTCTCAGATTGGTTCCTCGGAAACGATCAGGTTA

801 TCAACTGCAAAGACGTAATGGTTCCAAATTCAGTGTGTACGATGTGAGTA  
AGTTGACGTTTCTGCATTACCAAGGTTTAAGTCACACATGCTACACTCAT

851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGCACCTCCACCA  
ATAATTAGGGATAGTATCGAGAGTACCACATATCTACGCGTGGAGGTGGT

901 TCGTCACAGTTTTCTTTGCCCCTGGGTTTATGGGCTCCTAATTTTAATGC  
AGCAGTGTCAAAGAAACGGGGACCCAAATACCCGAGGATTAAATACG

951 TGATGTTTGTATGGATCCTGAGCCCATAGTGCATCGTAGGTCGAAATG  
ACTACAAACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTTAC

1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA  
CAGATACACAACACTACAATCCCTACCTTCTAAGGTGTTGCCTTTGCGTTAT

1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT  
GTCAACACCGGTACGTTTCAATTATGTCTACGTTTGTAGTCGAGACCTGAAA

1101 GAAAAGAGACAATACTATTCGATCTAATGGAAAGTGTTTAACTACTTACG  
CTTTTCTCTGTTATGATAAGCTAGATTACCTTTCACAAATTGATGAATGC

1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA  
CCATGTCAGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT

1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC  
TGACTACGGTGGGCGACCGTTTATACCCTATTACCTTGGTAGTATTTAGG

1251 CAGATCTAGTCTAGTTTTAGCAGCGACATCAGGGAACAGTGGTACCACAC  
GTCTAGATCAGATCAAATCGTCGCTGTAGTCCCTTGTACCATGGTGTG

1301 TTACAGTGCAAACCAACATTTATGCCGTTAGTCAAGGTTGGCTTCCTACT  
AATGTCACGTTTGGTTGTAAATACGGCAATCAGTTCCAACCGAAGGATGA

1351 AATAATACACAACCTTTTGTACAACCATTTGTTGGGCTATATGGTCTGTG  
TTATTATGTGTTGGAAAACAATGTTGGTAACAACCCGATATACCAGACAC

1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA  
GAACGTTTCGTTTATCACCTGTTTACATACCTATCTCCTGACATCGTCACTTT

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**FIGURE 34C (P3)**

1451 AGGCTGAACAAACAGTGGGCTCTTTATGCAGATGGTTCAATACGTCTCAG  
TCCGACTTGTTGTCACCCGAGAAATACGTCTACCAAGTTATGCAGGAGTC

1501 CAAAACCGAGATAATTGCCTTACAAGTGATTCTAATATACGGGAAACAGT  
GTTTTGGCTCTATTAAACGGAATGTTCACTAAGATTATATGCCCTTTGTCA

1551 TGTTAAGATCCTCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT  
ACAATTCTAGGAGAGAAACACCGGGACGTAGGAGACCGGTTGCTACCTACA

1601 TCAAGAATGATGGAACCATTTTAAATTTGTATAGTGGATTGGTGTTAGAT  
AGTTCTTACTACCTTGGTAAATTTAAACATATCACCTAACCACAATCTA

1651 GTGAGGCGATCGGATCCGAGCCTTAAACAAATCATTCTTTACCCTCTCCA  
CACTCCGCTAGCCTAGGCTCGGAATTTGTTTAGTAAGAAATGGGAGAGGT

1701 TGGTGACCCAAACCAAATATGGTTACCATTATTTTGATAGACAGATTACT  
ACCACTGGGTTTGGTTTATACCAATGGTAATAAACTATCTGTCTAATGA

1751 CTCTTGCAAGTGTGTGTGTCTCTGCCATGAAAATAGATGGCTTAAATAAAAA  
GAGAACGTCACACACACAGGACGGTACTTTTATCTACCGAATTTATTTTT

1801 GGACATTGTAAATTTTGTAAGTAAAGGACAGCAAGTTATATCGAATTCC  
CCTGTAACATTTAAAACATTGACTTTCCTGTCGTTCAATATAGCTTAAGG

1851 TGCAG  
ACGTC

Total number of bases is: 1855.

Sequence name: PAP270

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**FIGURE 34D**

**Amino acid sequence Comparison of Mutant Preproricin Linker  
region of MMP-2 to Wild Type**

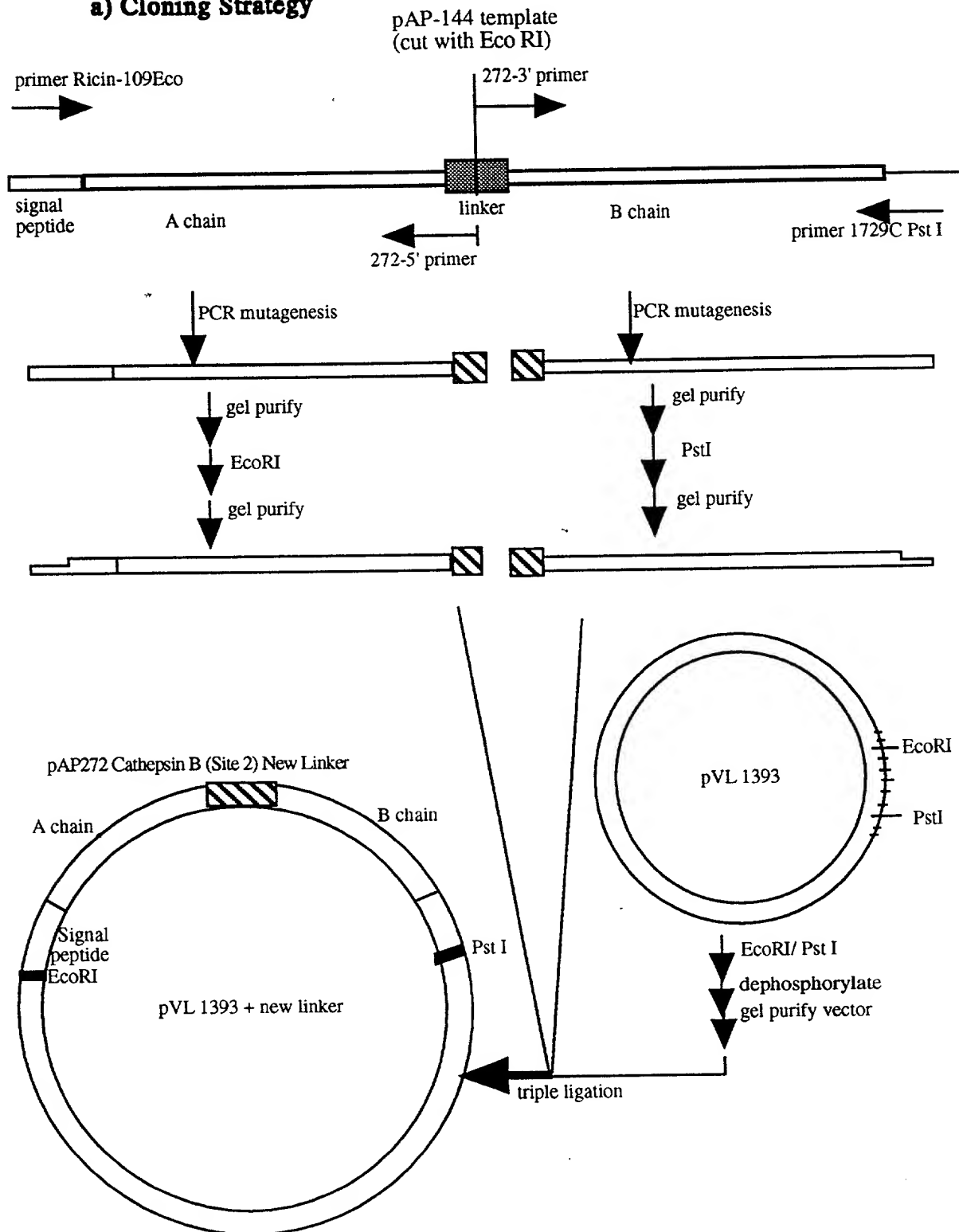
Wild type ricin linker:           A chain- S L L I R P V V P N F N -B chain

pAP-270 (MMP-2) linker:       A chain- S L P L G L W A P N F N -B chain

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**FIGURE 35A**

**PCR Mutagenesis of Preproricin Gene to Create A Variant Gene in Baculovirus Transfer Vector, pVL 1393**

**a) Cloning Strategy**

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## **FIGURE 35B**

### **Sequence of Cathepsin B (Site 2) Linker Region**

#### **WT preprocin linker**

primer 272-3'  
5' - AGGATGCCAAATTTTAATGCTGATGTTTGT -3'  
| \*\* \* \*  
-----TCTTTGCTTATAAGGCCA|GTGGTACCAAATTTTAAT-----  
-----AGAAACGAATATTCCGGT|CACCATGGTTTAAAATTA-----  
| \*\*\*\*\*  
3' -AGCAGTGTCAAAAGAAACGAATATCGATCT -5'  
primer 272-5'

1) PCR mutagenesis

2) Ligate with pVL1393

#### **pAP 272 linker**

**(Cathepsin B Site 2 variant)**

-----TCTTTGCTTATAGCTAGA|AGGATGCCTAATTTTAAT-----  
-----AGAAACGAATATCGATCT|TCCTACGGATTAAAATTA-----

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## FIGURE 35C (P1)

Sequence of pAP272 insert

```

          10          20          30          40          50
          |          |          |          |          |
1  GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT
   CTTAAGTACTTTGGCCCTCCTTTATGATAACATTATACCTACATACGTCA

51  GGCAACATGGCTTTGTTTTGGATCCACCTCAGGGTGGTCTTTCACATTAG
   CCGTTGTACCGAAACAAACCTAGGTGGAGTCCCACCAGAAAGTGTAAATC

101 AGGATAACAACATATTTCCCCAAACAATACCCAATTATAAACTTTACCACA
   TCCTATTGTTGTATAAGGGGTTTGTATGGGTAAATATTTGAAATGGTGT

151 GCGGGTGGCCACTGTGCAAAGCTACACAACTTTATCAGAGCTGTTTCGCGG
   CGCCACGGTGACACGTTTCGATGTGTTTGAAATAGTCTCGACAAGCGCC

201 TCGTTTAACAACCTGGAGCTGATGTGAGACATGATATACCAGTGTTGCCAA
   AGCAAATTGTTGACCTCGACTACACTCTGTACTATATGGTCACAACGGTT

251 ACAGAGTTGGTTTGCCTATAAACCAACGGTTTATTTTAGTTGAACTCTCA
   TGTCTCAACCAAACGGATATTTGGTTGCCAAATAAAATCAACTTGAGAGT

301 AATCATGCAGAGCTTTCTGTTACATTAGCGCTGGATGTCACCAATGCATA
   TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT

351 TGTGGTTCGGCTACCGTGCTGGAAATAGCGCATATTTCTTTCATCCTGACA
   ACACCAGCCGATGGCACGACCTTTATCGCGTATAAAGAAAGTAGGACTGT

401 ATCAGGAAGATGCAGAAGCAATCACTCATCTTTTCACTGATGTTCAAAT
   TAGTCCTTCTACGTCTTCGTTAGTGAGTAGAAAAGTGAAGTCAAGTTTAA

451 CGATATACATTTCGCTTTGGTGGTAATTATGATAGACTTGAACAACCTTGC
   GCTATATGTAAGCGGAAACCACCATTAATACTATCTGAAGTTGTTGAACG

501 TGGTAATCTGAGAGAAAATATCGAGTTGGGAAATGGTCCACTAGAGGAGG
   ACCATTAGACTCTCTTTTATAGCTCAACCCCTTACCAGGTGATCTCCTCC

551 CTATCTCAGCGCTTTATTATTACAGTACTGGTGGCACTCAGCTTCCAAC
   TATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA

601 CTGGCTCGTTTCCTTTATAATTTGCATCCAAATGATTTCAGAAGCAGCAAG
   GACCGAGCAAGGAAATATTAAACGTAGGTTTACTAAAGTCTTCGTCGTTT

651 ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA
   TAAGGTTATATAACTCCCTCTTTACGCGTGCTCTTAATCCATGTTGGCCT

```

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**FIGURE 35C (P2)**

701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGGAGA  
CTAGACGTGGTCTAGGATCGCATTAAATGTGAACTCTTATCAACCCCTCT

751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTTGCTAGTCCAAT  
GAAAGGTGACGTTAAGTTCTCAGATTGGTTCCTCGGAAACGATCAGGTTA

801 TCAACTGCAAAGACGTAATGGTTCCAAATTCAGTGTGTACGATGTGAGTA  
AGTTGACGTTTCTGCATTACCAAGGTTTAAGTCACACATGCTACACTCAT

851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGCACCTCCACCA  
ATAATTAGGGATAGTATCGAGAGTACCACATATCTACGCGTGGAGGTGGT

901 TCGTCACAGTTTTCTTTGCTTATAGCTAGAAGGATGCCTAATTTTAATGC  
AGCAGTGTCAAAGAAAGGAATATCGATCTTCCTACGGATTAAAATTACG

951 TGATGTTTGTATGGATCCTGAGCCCATAGTGCGTATCGTAGGTGCAAATG  
ACTACAAACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTTAC

1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA  
CAGATACACAACACTACAATCCCTACCTTCTAAGGTGTTGCCTTTGCGTTAT

1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT  
GTCAACACCGGTACGTTTCAGATTATGTCTACGTTTAGTCGAGACCTGAAA

1101 GAAAAGAGACAATACTATTCGATCTAATGGAAAGTGTTTAACTACTTACG  
CTTTTCTCTGTTATGATAAGCTAGATTACCTTTCACAAATTGATGAATGC

1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA  
CCATGTCAGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT

1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC  
TGACTACGGTGGGCGACCGTTTATACCCTATTACCTTGGTAGTATTTAGG

1251 CAGATCTAGTCTAGTTTTAGCAGCGACATCAGGGAACAGTGGTACCACAC  
GTCTAGATCAGATCAAATCGTCGCTGTAGTCCCTTGTCACCATGGTGTG

1301 TTACAGTGCAAACCAACATTTATGCCGTTAGTCAAGGTGGCTTCCTACT  
AATGTCACGTTTGGTTGTAAATACGGCAATCAGTTCCAACCGAAGGATGA

1351 AATAATACACAACCTTTTGTTACAACCATTTGTTGGGCTATATGGTCTGTG  
TTATTATGTGTTGAAAACAATGTTGGTAACAACCCGATATAACCAGACAC

1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA  
GAACGTTGTTTTATCACCTGTTTCATACCTATCTCCTGACATCGTCACTTT

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**FIGURE 35C (P3)**

1451 AGGCTGAACAACAGTGGGCTCTTTATGCAGATGGTTCAATACGTCCTCAG  
TCCGACTTGTTGTCACCCGAGAAATACGTCTACCAAGTTATGCAGGAGTC

1501 CAAAACCGAGATAATTGCCTTACAAGTGATTCTAATATACGGGAAACAGT  
GTTTTGGCTCTATTAACGGAATGTTCACTAAGATTATATGCCCTTTGTCA

1551 TGTTAAGATCCTCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT  
ACAATTCTAGGAGAGAACACCGGGACGTAGGAGACCGGTTGCTACCTACA

1601 TCAAGAATGATGGAACCATTTTAAATTTGTATAGTGGATTGGTGTTAGAT  
AGTTCTTACTACCTTGGTAAAATTTAAACATATCACCTAACCACAATCTA

1651 GTGAGGCGATCGGATCCGAGCCTTAAACAAATCATTCTTTACCCTCTCCA  
CACTCCGCTAGCCTAGGCTCGGAATTTGTTTAGTAAGAAATGGGAGAGGT

1701 TGGTGACCCAAACCAAATATGGTTACCATTATTTTGATAGACAGATTACT  
ACCACTGGGTTTGGTTTATACCAATGGTAATAAACTATCTGTCTAATGA

1751 CTCTTGCAGTGTGTGTGTCCTGCCATGAAAATAGATGGCTTAAATAAAAA  
GAGAACGTCACACACACAGGACGGTACTTTTATCTACCGAATTTATTTTT

1801 GGACATTGTAAATTTTGTAAGTAAAGGACAGCAAGTTATATCGAATTCC  
CCTGTAACATTTAAACATTGACTTTCCTGTCGTTCAATATAGCTTAAGG

1851 TGCAG  
ACGTC

Total number of bases is: 1855.

Sequence name: PAP272

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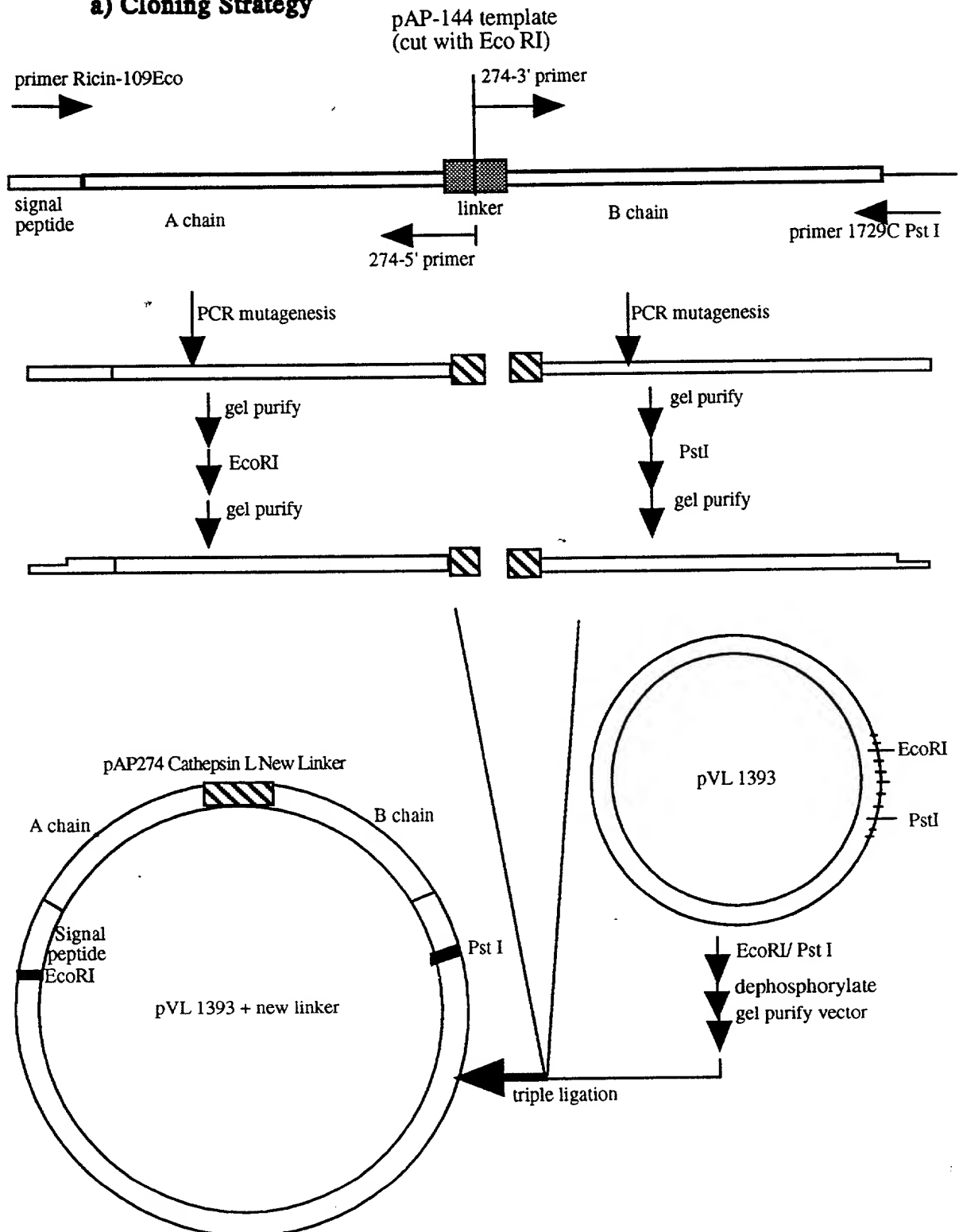
**FIGURE 35D**

**Amino acid sequence Comparison of Mutant Preproricin Linker  
region of Cathepsin B Site 2 to Wild Type**

Wild type ricin linker:           A chain- S L L I R P V V P N F N -B chain

pAP-272 (Cathepsin B 2) linker: A chain- S L L I A R R M P N F N -B chain

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**FIGURE 36A****PCR Mutagenesis of Preproricin Gene to Create A Variant Gene in Baculovirus Transfer Vector, pVL 1393****a) Cloning Strategy**

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## FIGURE 36B

### Sequence of Cathepsin L Linker Region

#### WT preprocin linker

```

                                primer 274-3'
                                5' - TCATGGGCTAATTTTAATGCTGATGTTTGT -3'
                                | ***** *
-----TCTTTGCTTATAAGGCCA|GTGGTACCAAATTTTAAT-----
-----AGAAACGAATATTCCGGT|CACCATGGTTTAAAATTA-----
                                *** **
3' -AGCAGTGTCAAAAGAAACGAATATAAGGCC -5'
                                primer 274-5'
```

1) PCR mutagenesis

2) Ligate with pVL1393

#### pAP 274 linker (Cathepsin L variant)

```
-----TCTTTGCTTATATTCCGG|TCATGGGCTAATTTTAAT-----
-----AGAAACGAATATAAGGCC|AGTACCCGATTAAAATTA-----
```

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## FIGURE 36C (P1)

Sequence of pAP274 insert

```

      10      20      30      40      50
      |      |      |      |      |
1  GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT
   CTTAAGTACTTTGGCCCTCCTTTATGATAACATTATACCTACATACGTCA

51  GGCAACATGGCTTTGTTTTGGATCCACCTCAGGGTGGTCTTTCACATTAG
   CCGTTGTACCGAAACAAACCTAGGTGGAGTCCCACCAGAAAGTGTAAATC

101 AGGATAACAACATATTCCCCAAACAATACCCAATTATAAACTTTACCACA
   TCCTATTGTTGTATAAGGGGTTTGTATGGGTAAATATTTGAAATGGTGT

151 GCGGGTGCCACTGTGCAAAGCTACACAACTTTATCAGAGCTGTTGCGGG
   CGCCACGGTGACACGTTTCGATGTGTTTGAAATAGTCTCGACAAGCGCC

201 TCGTTTAACAACCTGGAGCTGATGTGAGACATGATATACCAGTGTTGCCAA
   AGCAAATTGTTGACCTCGACTACACTCTGTACTATATGGTCACAACGGTT

251 ACAGAGTTGGTTTGCCCTATAAACCAACGGTTTATTTTAGTTGAACTCTCA
   TGTCTCAACCAAACGGATATTTGGTTGCCAAATAAAATCAACTTGAGAGT

301 AATCATGCAGAGCTTTCTGTTACATTAGCGCTGGATGTCACCAATGCATA
   TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTACGTAT

351 TGTGGTCGGCTACCGTGCTGGAAATAGCGCATATTTCTTTCATCCTGACA
   ACACCAGCCGATGGCACGACCTTTATCGCGTATAAAGAAAGTAGGACTGT

401 ATCAGGAAGATGCAGAAGCAATCACTCATCTTTTCACTGATGTTCAAAT
   TAGTCCTTCTACGTCTTCGTTAGTGAGTAGAAAAGTGAATAAAGTTTAA

451 CGATATACATTGCGCTTTGGTGGTAATTATGATAGACTTGAACAACCTTGC
   GCTATATGTAAGCGGAAACCACCATTAATACTATCTGAACTTGTTGAACG

501 TGGTAATCTGAGAGAAAATATCGAGTTGGGAAATGGTCCACTAGAGGAGG
   ACCATTAGACTCTCTTTTATAGCTCAACCCTTTACCAGGTGATCTCCTCC

551 CTATCTCAGCGCTTTATTATTACAGTACTGGTGGCACTCAGCTTCCAAT
   GATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA

601 CTGGCTCGTTCCTTTATAATTTGCATCCAAATGATTTCAGAAGCAGCAAG
   GACCGAGCAAGGAAATATTAAACGTAGGTTTACTAAAGTCTTCGTCGTTTC

651 ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA
   TAAGGTTATATAACTCCCTCTTTACGCGTGCTCTTAATCCATGTTGGCCT
```

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**FIGURE 36C (P2)**

701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGGAGA  
CTAGACGTGGTCTAGGATCGCATTAAATGTGAACTCTTATCAACCCCTCT

751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTTGCTAGTCCAAT  
GAAAGGTGACGTTAAGTTCTCAGATTGGTTCCTCGGAAACGATCAGGTTA

801 TCAACTGCAAAGACGTAATGGTTCCAAATTCAGTGTGTACGATGTGAGTA  
AGTTGACGTTTCTGCATTACCAAGGTTTAAGTCACACATGCTACACTCAT

851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGCACCTCCACCA  
ATAATTAGGGATAGTATCGAGAGTACCACATATCTACGCGTGGAGGTGGT

901 TCGTCACAGTTTTCTTTGCTTATATTCCGGTCATGGGCTAATTTTAATGC  
AGCAGTGTCAAAGAAAGGAATATAAGGCCAGTACCCGATTAAAATTACG

951 TGATGTTTGTATGGATCCTGAGCCCATAGTGCGTATCGTAGGTCGAAATG  
ACTACAAACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTTAC

1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA  
CAGATACACAACACTACAATCCCTACCTTCTAAGGTGTTGCCTTTGCGTTAT

1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT  
GTCAACACCGGTACGTTTCAAGTATGTCTACGTTTAGTCGAGACCTGAAA

1101 GAAAAGAGACAATACTATTCGATCTAATGGAAAGTGTTTAACTACTTACG  
CTTTTCTCTGTTATGATAAGCTAGATTACCTTTCACAAATTGATGAATGC

1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA  
CCATGTCAGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT

1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC  
TGACTACGGTGGGCGACCGTTTATACCCTATTACCTTGGTAGTATTTAGG

1251 CAGATCTAGTCTAGTTTTAGCAGCGACATCAGGGAACAGTGGTACCACAC  
GTCTAGATCAGATCAAATCGTCGCTGTAGTCCCTTGTCCACCATGGTGTG

1301 TTACAGTGCAAACCAACATTTATGCCGTTAGTCAAGGTTGGCTTCCTACT  
AATGTCACGTTTGGTTGTAAATACGGCAATCAGTTCCAACCGAAGGATGA

1351 AATAATACACAACCTTTTGTTACAACCATTTGTTGGGCTATATGGTCTGTG  
TTATTATGTGTTGGAAAACAATGTTGGTAACAACCCGATATACCAGACAC

1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA  
GAACGTTTCGTTTATCACCTGTTTACACCTATCTCCTGACATCGTCACTTT

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**FIGURE 36C (P3)**

1451 AGGCTGAACAACAGTGGGCTCTTTATGCAGATGGTTCAATACGTCCTCAG  
TCCGACTTGTTGTCACCCGAGAAATACGTCTACCAAGTTATGCAGGAGTC

1501 CAAAACCGAGATAATTGCCTTACAAGTGATTCTAATATACGGGAAACAGT  
GTTTTGGCTCTATTAACGGAATGTTCACTAAGATTATATGCCCTTTGTCA

1551 TGTTAAGATCCTCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT  
ACAATTCTAGGAGAGAACACCGGGACGTAGGAGACCGGTTGCTACCTACA

1601 TCAAGAATGATGGAACCATTTTAAATTTGTATAGTGGATTGGTGTTAGAT  
AGTTCTTACTACCTTGGTAAAATTTAAACATATCACCTAACCACAATCTA

1651 GTGAGGCGATCGGATCCGAGCCTTAAACAAATCATTCTTTACCCTCTCCA  
CACTCCGCTAGCCTAGGCTCGGAATTTGTTTAGTAAGAAATGGGAGAGGT

1701 TGGTGACCCAAACCAAATATGGTTACCATTATTTTGATAGACAGATTACT  
ACCACTGGGTTTGGTTTATACCAATGGTAATAAACTATCTGTCTAATGA

1751 CTCTTGCAGTGTGTGTGTCCTGCCATGAAAATAGATGGCTTAAATAAAAA  
GAGAACGTCACACACACAGGACGGTACTTTTATCTACCGAATTTATTTTT

1801 GGACATTGTAAATTTTGTAACTGAAAGGACAGCAAGTTATATCGAATTCC  
CCTGTAAACATTTAAACATTGACTTTCCTGTCGTTCAATATAGCTTAAGG

1851 TGCAG  
ACGTC

Total number of bases is: 1855.

Sequence name: PAP274



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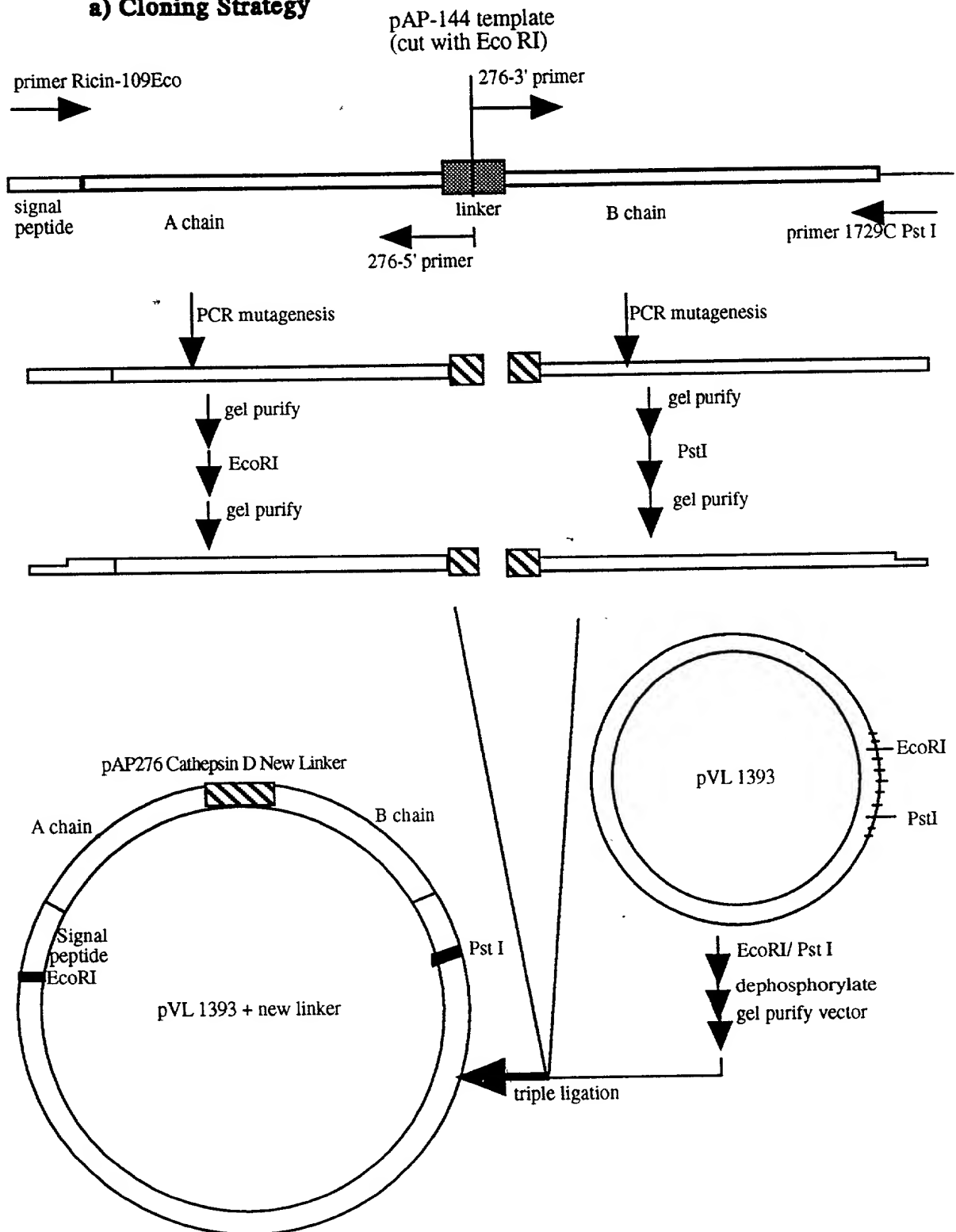
**FIGURE 36D**

**Amino acid sequence Comparison of Mutant Preproricin Linker  
region of Cathepsin L to Wild Type**

Wild type ricin linker:      ,    A chain- S L L I R P V V P N F N -B chain

pAP-274 (Cathepsin L)linker:    A chain- S L L I F R S W A N F N -B chain

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**FIGURE 37A****PCR Mutagenesis of Preproricin Gene to Create A Variant Gene in Baculovirus Transfer Vector, pVL 1393****a) Cloning Strategy**

**FIGURE 37B**

### Sequence of Cathepsin D Linker Region

**WT preprocin linker**

```

          primer 276-3'
          5'- ACTGTTATTGTTATCACCGCTGATGTTTGT -3'
          ***   * * * * *
-----TCTTTGCTTATAAGGCCA|GTGGTACCAAATTTTAAT-----
-----AGATAACGAATATTCCGG|CACCATGGTTTAAAATTA-----
          ****   * * * * *
          3'-AGCAGTGTCAAAGACCACAACAGTAGCGA -5'
          primer 276-5'

```

- 1) PCR mutagenesis
- 2) Ligate with pVL1393

**pAP 276 linker  
(Cathepsin D variant)**

-----TCTGGTGTGTGTCATCGCT|ACTGTTATTGTTATCACC-----  
-----AGACCACAACAGTAGCGA|TGACAATAACAATAGTGG-----

**FIGURE 37C (P1)**

Sequence of pAP276 insert

10 20 30 40 50

1 GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT  
CTTAAGTACTTTGGCCCTCCTTTATGATAACATTATACCTACATACGTCA

51 GGCAACATGGCTTTGTTTGGATCCACCTCAGGGTGGTCTTTCACATTAG  
CCGTTGTACCGAAACAAAACCTAGGTGGAGTCCCACCAGAAAGTGTATC

101 AGGATAACAACATATTCCCCAAACAATACCCAATTATAAACTTTACCACA  
TCCTATTGTTGTATAAGGGGTTTGTATGGGTAAATATTTGAAATGGTGT

151 GCGGGTGCCACTGTGCAAAGCTACACAACTTTATCAGAGCTGTTTCGCGG  
CGCCACGGTGACACGTTTCGATGTGTTTGAATAGTCTCGACAAGCGCC

201 TCGTTTAAACAACTGGAGCTGATGTGAGACATGATATACAGTGTTGCCAA  
AGCAAATTGTTGACCTCGACTACACTCTGTACTATATGGTCACAACGGTT

251 ACAGAGTTGGTTTGCCTATAAACCAACGGTTTATTTTAGTTGAACTCTCA  
TGTCTCAACCAAACGGATATTTGGTTGCCAAATAAAATCAACTTGAGAGT

301 AATCATGCAGAGCTTCTGTACATTAGCGCTGGATGTCACCAATGCATA  
TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT

351 TGTGGTCGGCTACCGTGCTGGAAATAGCGCATATTTCTTTCATCCTGACA  
ACACCAGCCGATGGCACGACCTTTATCGCGTATAAAGAAAGTAGGACTGT

401 ATCAGGAAGATGCAGAAGCAATCACTCATCTTTTCACTGATGTTCAAAT  
TAGTCCTTCTACGTCTTCGTTAGTGAGTAGAAAAGTGACTACAAGTTTTTA

451 CGATATACATTTCGCCTTTGGTGGTAATTATGATAGACTTGAACAACTTGC  
GCTATATGTAAGCGGAAACCACCATTAATACTATCTGAACTTGTTGAACG

501 TGGTAATCTGAGAGAAAATATCGAGTTGGGAAATGGTCCACTAGAGGAGG  
ACCATTAGACTCTCTTTTATAGCTCAACCCTTTACCAGGTGATCTCCTCC

551 CTATCTCAGCGCTTTATTATTACAGTACTGGTGGCACTCAGCTTCCAACT  
GATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA

601 CTGGCTCGTTCCTTTATAATTTGCATCCAAATGATTTCAGAAGCAGCAAG  
GACCGAGCAAGGAAATATTAAACGTAGGTTTACTAAAGTCTTCGTCTGTTT

651 ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA  
TAAGGTTATATAACTCCCTCTTTACGCGTGCTCTTAATCCATGTTGGCCT

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**FIGURE 37C (P2)**

701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGGAGA  
CTAGACGTGGTCTAGGATCGCATTAAATGTGAACTCTTATCAACCCCCTCT

751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTTGCTAGTCCAAT  
GAAAGGTGACGTTAAGTTCTCAGATTGGTTCCTCGGAAACGATCAGGTTA

801 TCAACTGCAAAGACGTAATGGTTCCAAATTCAGTGTGTACGATGTGAGTA  
AGTTGACGTTTCTGCATTACCAAGGTTTAAGTCACACATGCTACACTCAT

851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGCACCTCCACCA  
ATAATTAGGGATAGTATCGAGAGTACCACATATCTACGCGTGGAGGTGGT

901 TCGTCACAGTTTTCTGGTGTTCATCGCTACTGTTATTGTTATCACCGC  
AGCAĜTGTCAAAAGACCACAACAGTAGCGATGACAATAACAATAGTGGCG

951 TGATGTTTGTATGGATCCTGAGCCCATAGTGCGTATCGTAGGTGCAAATG  
ACTACAAACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTTAC

1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA  
CAGATACACAACACTACAATCCCTACCTTCTAAGGTGTTGCCTTTGCGTTAT

1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT  
GTCAACACCGGTACGTTTCAGATTATGTCTACGTTTAGTCGAGACCTGAAA

1101 GAAAAGAGACAATACTATTTCGATCTAATGGAAAGTGTTTAACTACTTACG  
CTTTTCTCTGTTATGATAAGCTAGATTACCTTTTACAAATTGATGAATGC

1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA  
CCATGTCAGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT

1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC  
TGACTACGGTGGGCGACCGTTTATACCCTATTACCTTGGTAGTATTTAGG

1251 CAGATCTAGTCTAGTTTTAGCAGCGACATCAGGGAACAGTGGTACCACAC  
GTCTAGATCAGATCAAAATCGTCGCTGTAGTCCCĤTGTCAACCATGGTGTG

1301 TTACAGTGCAAACCAACATTTATGCCGTTAGTCAAGGTTGGCTTCCTACT  
AATGTCACGTTTGGTTGTAAATACGGCAATCAGTTCCAACCGAAGGATGA

1351 AATAATACACAACCTTTTGTTACAACCATTTGTTGGGCTATATGGTCTGTG  
TTATTATGTGTTGGAAAACAATGTTGGTAACAACCCGATATAACCAGACAC

1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA  
GAACGTTCTGTTTATCACCTGTTTCATACCTATCTCCTGACATCGTCACTTT

004440"1511560

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**FIGURE 37C (P3)**

1451 AGGCTGAACAACAGTGGGCTCTTTATGCAGATGGTTCAATACGTCCTCAG  
TCCGACTTGTTGTCACCCGAGAAATACGTCTACCAAGTTATGCAGGAGTC

1501 CAAAACCGAGATAATTGCCTTACAAGTGATTCTAATATACGGGAAACAGT  
GTTTTGGCTCTATTAACGGAATGTTCACTAAGATTATATGCCCTTTGTCA

1551 TGTTAAGATCCTCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT  
ACAATTCTAGGAGAGAACACCGGGACGTAGGAGACCGGTTGCTACCTACA

1601 TCAAGAATGATGGAACCATTTTAAATTTGTATAGTGGATTGGTGTTAGAT  
AGTTCTTACTACCTTGGTAAAATTTAAACATATCACCTAACCACAATCTA

1651 GTGAGGCGATCGGATCCGAGCCTTAAACAAATCATTCTTTACCCTCTCCA  
CACTCCGCTAGCCTAGGCTCGGAATTTGTTTAGTAAGAAATGGGAGAGGT

1701 TGGTGACCCAAACCAAATATGGTTACCATTATTTTGATAGACAGATTACT  
ACCACTGGGTTTGGTTTATACCAATGGTAATAAACTATCTGTCTAATGA

1751 CTCTTGCAGTGTGTGTGTCCTGCCATGAAAATAGATGGCTTAAATAAAAA  
GAGAACGTCACACACACAGGACGGTACTTTTATCTACCGAATTTATTTTT

1801 GGACATTGTAAATTTTGTAACTGAAAGGACAGCAAGTTATATCGAATTCC  
CCTGTAAACATTTAAACATTGACTTTCCTGTCGTTCAATATAGCTTAAGG

1851 TGCAG  
ACGTC

Total number of bases is: 1855.

Sequence name: PAP276

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**FIGURE 37D**

**Amino acid sequence Comparison of Mutant Preproricin Linker  
region of Cathepsin D to Wild Type**

Wild type ricin linker:           A chain- S L L I R P V V P N F N -B chain

pAP-276 (Cathepsin D) linker: A chain- S G V V I A T V I V I T -B chain

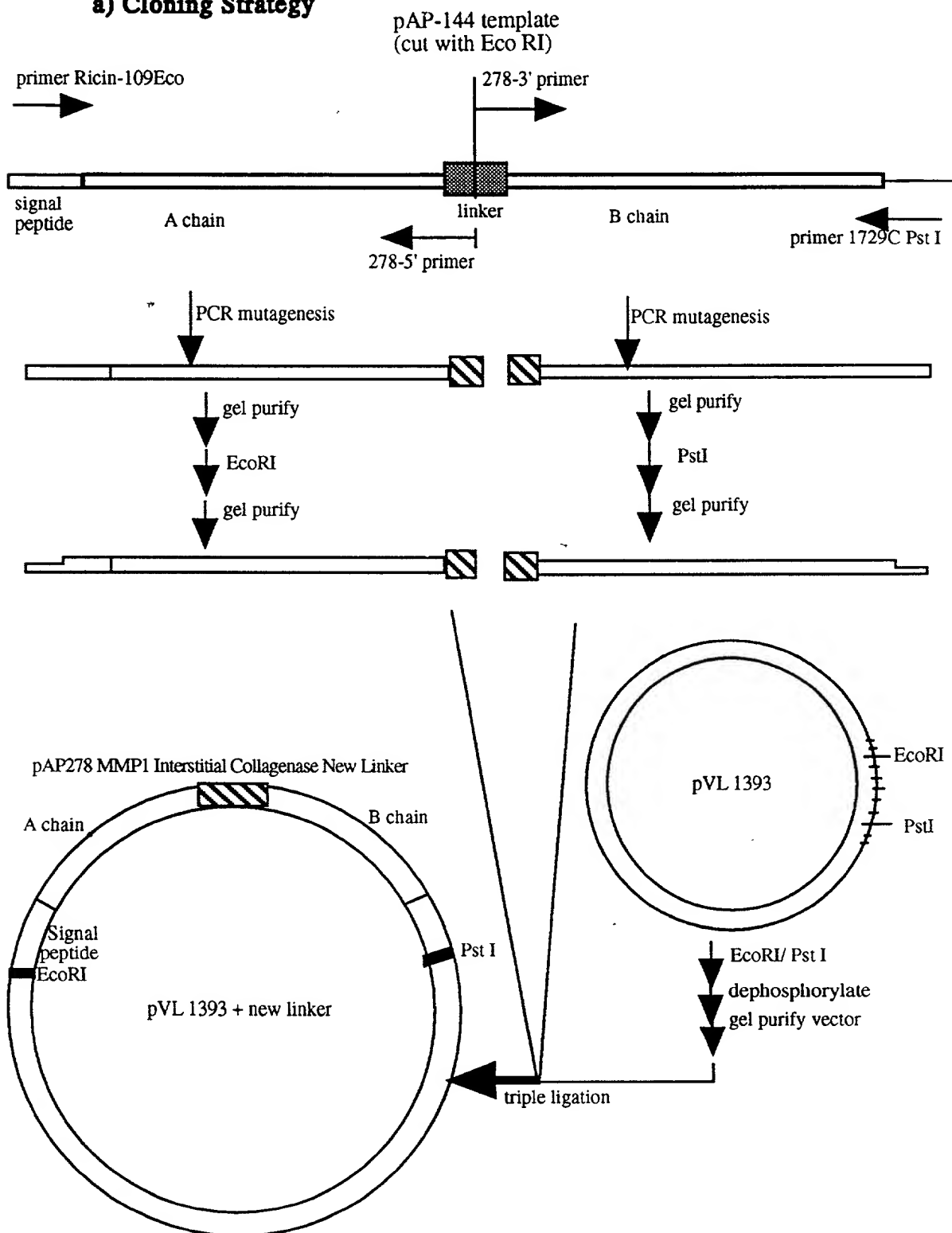
004440"TS1560

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# FIGURE 38A

PCR Mutagenesis of Preproricin Gene to Create A Variant Gene in Baculovirus Transfer Vector, pVL 1393

## a) Cloning Strategy





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## **FIGURE 38B**

### **Sequence of MMP-1 (Interstitial collagenase) Linker Region**

#### **WT preprocin linker**

primer 278-3'  
5' - ATTTGGGGACAGTTTAATGCTGATGTTTGT -3'  
\* \* \* \* \*  
-----TCTTTGCTTATAAGGCCA|GTGGTACCAAATTTTAAT-----  
-----AGAAACGAATATTCCGGT|CACCATGGTTTAAAATTA-----  
\*\* \* \* \* \* \*  
3' -AGCAGTGTCAAAAGAAACCCAGGAGTTCCG -5'  
primer 278-5'

1) PCR mutagenesis

2) Ligate with pVL1393

#### **pAP 278 linker (MMP-1 variant)**

-----TCTTTGGGTCCTCAAGGC|ATTTGGGGACAGTTTAAT-----  
-----AGAAACCCAGGAGTTCCG|TAAACCCCTGTCAAATTA-----

00440" F5F550

**FIGURE 38C (P1)**

Sequence of pAP278 insert

10 20 30 40 50

1 GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT  
CTTAAGTACTTTGGCCCTCCTTTATGATAACATTATACCTACATACGTCA

51 GGCAACATGGCTTTGTTTTGGATCCACCTCAGGGTGGTCTTTCACATTAG  
CCGTTGTACCGAAACAAAACCTAGGTGGAGTCCCACCAGAAAGTGTAATC

101 AGGATAACAACATATTCCCCAAACAATACCCAATTATAAACTTTACCACA  
TCCTATTGTTGTATAAGGGGTTTGTATTATGGGTAAATATTTGAAATGGTGT

151 GCGGGTGCCACTGTGCAAAGCTACACAACTTTATCAGAGCTGTTTCGCGG  
CGCCACGGTGACACGTTTTCGATGTGTTTGAAATAGTCTCGACAAGCGCC

201 TCGTTTAAACAACTGGAGCTGATGTGAGACATGATATACCAGTGTTGCCAA  
AGCAAATTGTTGACCTCGACTACACTCTGTACTATATGGTCACAACGGTT

251 ACAGAGTTGGTTTGCCTATAAACCAACGGTTTATTTTAGTTGAACTCTCA  
TGTCTCAACCAAACGGATATTTGGTTGCCAAATAAAATCAACTTGAGAGT

301 AATCATGCAGAGCTTTCTGTACATTAGCGCTGGATGTCACCAATGCATA  
TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT

351 TGTGGTCGGCTACCGTGCTGGAAATAGCGCATATTTCTTTCATCCTGACA  
ACACCAGCCGATGGCACGACCTTTATCGCGTATAAAGAAAGTAGGACTGT

401 ATCAGGAAGATGCAGAAGCAATCACTCATCTTTTCACTGATGTTCAAAT  
TAGTCCTTCTACGTCTTCGTTAGTGAGTAGAAAAGTGACTACAAGTTTTTA

451 CGATATACATTTCGCCTTTGGTGGTAATTATGATAGACTTGAACAACCTTGC  
GCTATATGTAAGCGGAAACCACCATTAATACTATCTGAACTTGTTGAACG

501 TGGTAATCTGAGAGAAAATATCGAGTTGGGAAATGGTCCACTAGAGGAGG  
ACCATTAGACTCTCTTTTATAGCTCAACCCTTTACCAGGTGATCTCCTCC

551 CTATCTCAGCGCTTTATTATTACAGTACTGGTGGCACTCAGCTTCCAAC  
TATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA

601 CTGGCTCGTTCCTTTATAATTTGCATCCAAATGATTTTCTGAGAGCAGCAAG  
GACCGAGCAAGGAAATATTAAACGTAGGTTTACTAAAGTCTTCGTCGTTT

651 ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA  
TAAGGTTATATAACTCCCTCTTTACGCGTGCTCTTAATCCATGTTGGCCT

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**FIGURE 38C (P2)**

701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGGAGA  
CTAGACGTGGTCTAGGATCGCATTAAATGTGAACTCTTATCAACCCCCTCT

751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTTGCTAGTCCAAT  
GAAAGGTGACGTTAAGTTCTCAGATTGGTTCCTCGGAAACGATCAGGTTA

801 TCAACTGCAAAGACGTAATGGTTCCAAATTCAGTGTGTACGATGTGAGTA  
AGTTGACGTTTCTGCATTACCAAGGTTTAAGTCACACATGCTACACTCAT

851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGCACCTCCACCA  
ATAATTAGGGATAGTATCGAGAGTACCACATATCTACGCGTGGAGGTGGT

901 TCGTCACAGTTTTCTTTGGGTCCCTCAAGGCATTTGGGGACAGTTTAATGC  
AGCAGTGTCAAAGAAACGCAGGAGTTCCGTAAACCCCTGTCAAATTACG

951 TGATGTTTGTATGGATCCTGAGCCCATAGTGCGTATCGTAGGTGCAAATG  
ACTACAAACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTTAC

1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGAAACGCAATA  
CAGATACACAACCTACAATCCCTACCTTCTAAGGTGTTGCCTTTGCGTTAT

1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT  
GTCAACACCGGTACGTTTACGATTATGTCTACGTTTAGTCGAGACCTGAAA

1101 GAAAAGAGACAATACTATTCGATCTAATGGAAAGTGTTTAACTACTTACG  
CTTTTCTCTGTTATGATAAGCTAGATTACCTTTCACAAATTGATGAATGC

1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA  
CCATGTCAGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT

1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC  
TGACTACGGTGGGCGACCGTTTATACCTATTACCTTGGTAGTATTTAGG

1251 CAGATCTAGTCTAGTTTTAGCAGCGACATCAGGGAACAGTGGTACCACAC  
GTCTAGATCAGATCAAAATCGTCGCTGTAGTCCCTTGTCAACCATGGTGTG

1301 TTACAGTGCAAACCAACATTTATGCCGTTAGTCAAGGTTGGCTTCCTACT  
AATGTCACGTTTGGTTGTAAATACGGCAATCAGTTCCAACCGAAGGATGA

1351 AATAATACACAACCTTTTGTACAAACCATTGTTGGGCTATATGGTCTGTG  
TTATTATGTGTTGGAAAACAATGTTGGTAACAACCCGATATACCAGACAC

1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA  
GAACGTTTCGTTTATCACCTGTTTCATACCTATCTCCTGACATCGTCACTTT

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**FIGURE 38C (P3)**

1451 AGGCTGAACAACAGTGGGCTCTTTATGCAGATGGTTCAATACGTCCTCAG  
TCCGACTTGTTGTCACCCGAGAAATACGTCTACCAAGTTATGCAGGAGTC

1501 CAAAACCGAGATAATTGCCTTACAAGTGATTCTAATATACGGGAAACAGT  
GTTTTGGCTCTATTAAACGGAATGTTCACTAAGATTATATGCCCTTTGTCA

1551 TGTTAAGATCCTCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT  
ACAATTCTAGGAGAGAACACCGGGACGTAGGAGACCGGTTGCTACCTACA

1601 TCAAGAATGATGGAACCATTTTAAATTTGTATAGTGGATTGGTGTTAGAT  
AGTTCTTACTACCTTGGTAAAATTTAAACATATCACCTAACCACAATCTA

1651 GTGAGGCGATCGGATCCGAGCCTTAAACAAATCATTCTTTACCCTCTCCA  
CACTCCGCTAGCCTAGGCTCGGAATTTGTTTAGTAAGAAATGGGAGAGGT

1701 TGGTGACCCAAACCAAATATGGTTACCATTATTTTGATAGACAGATTACT  
ACCACTGGGTTTGGTTTATACCAATGGTAATAAACTATCTGTCTAATGA

1751 CTCTTGCACTGTGTGTGTCCTGCCATGAAAATAGATGGCTTAAATAAAAA  
GAGAACGTCACACACACAGGACGGTACTTTTATCTACCGAATTTATTTTT

1801 GGACATTGTAAATTTTGTAACTGAAAGGACAGCAAGTTATATCGAATTCC  
CCTGTAAACATTTAAACATTGACTTTCCTGTCGTTCAATATAGCTTAAGG

1851 TGCAG  
ACGTC

Total number of bases is: 1855.

Sequence name: PAP278

004440-151550

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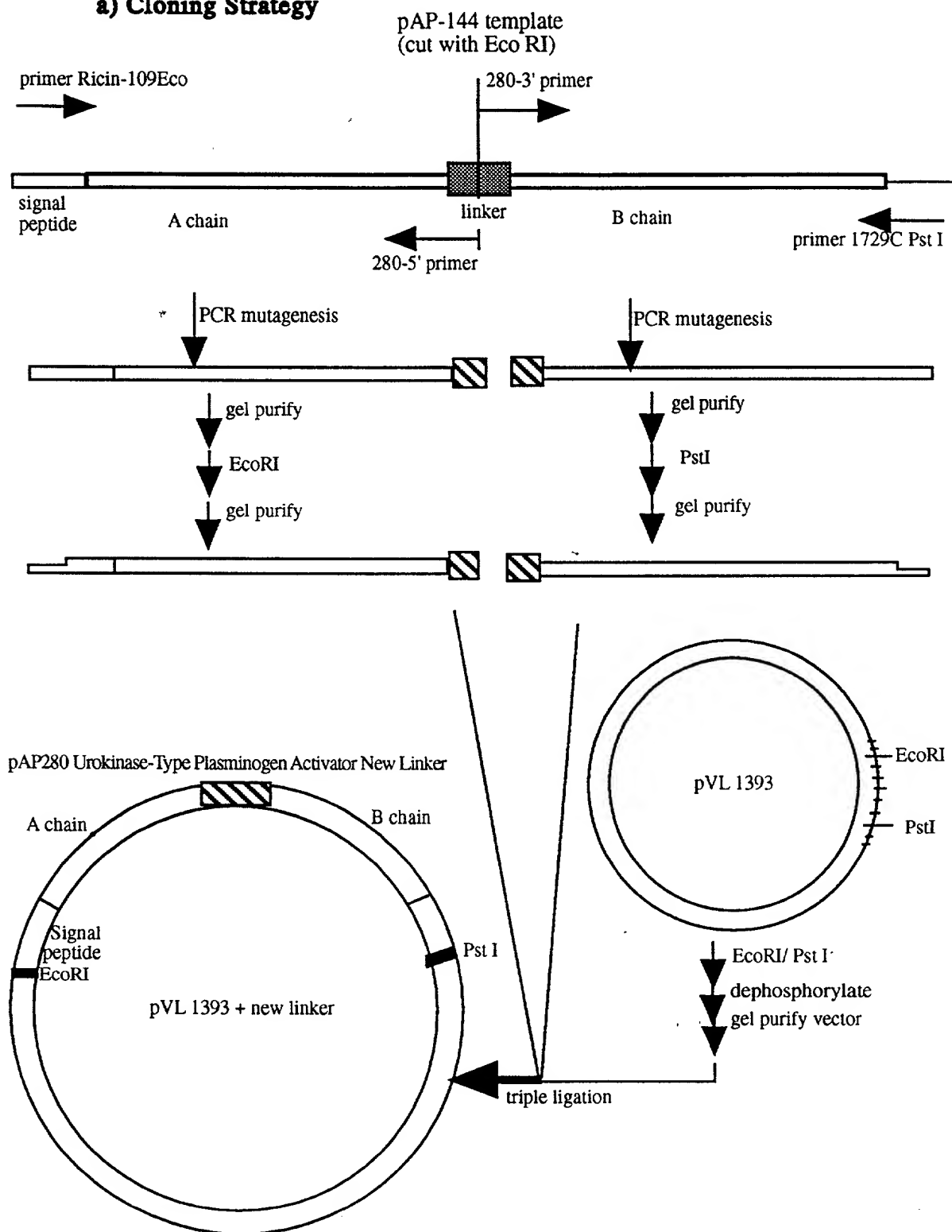
**FIGURE 38D**

**Figure 38. d) Amino acid sequence Comparison of Mutant Preproricin Linker region of MMP-1 (Interstitial collagenase) to Wild Type**

Wild type ricin linker:      A chain- S L L I R P V V P N F N -B chain

pAP-278 (MMP-1) linker:      A chain- S L G P Q G I W G Q F N -B chain

00440"TSF360

**FIGURE 39A****PCR Mutagenesis of Preproricein Gene to Create A Variant Gene in Baculovirus Transfer Vector, pVL 1393****a) Cloning Strategy**

**WT preprocin linker**

★      ★ ★ ★ ★ ★ ★ ★      ★      ★ ★ ★

primer 280-5'

- 1) PCR mutagenesis
- 2) Ligate with pVL1393

-----  
 -----AAAAAATCCCCTGGAAGA|GTTGTGGTGGCTCTGTA-----  
 -----TTTTTTAGGGGACCTTCT|CAACAGCCACCGAGACAT-----

**FIGURE 39C (P1)**

Sequence of pAP280 insert

10 20 30 40 50

1 GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT  
CTTAAGTACTTTGGCCCTCCTTTATGATAACATTATACCTACATACGTCA

51 GGCAACATGGCTTTGTTTTGGATCCACCTCAGGGTGGTCTTTCACATTAG  
CCGTTGTACCGAAACAAAACCTAGGTGGAGTCCCACCAGAAAGTGTAAATC

101 AGGATAACAACATATTCCTCCAAACAATACCCAATTATAAACTTTACCACA  
TCCTATTGTTGTATAAGGGGTTTGTATGGGTAAATATTTGAAATGGTGT

151 GCGGGTGCCACTGTGCAAAGCTACACAACTTTATCAGAGCTGTTTCGCGG  
CGCCACGGTGACACGTTTCGATGTGTTTGAAATAGTCTCGACAAGCGCC

201 TCGTTTAAACAACTGGAGCTGATGTGAGACATGATATACCAGTGTTCGCAA  
AGCAAATTGTTGACCTCGACTACACTCTGTACTATATGGTCACAACGGTT

251 ACAGAGTTGGTTTGCCTATAAACCAACGGTTTATTTTAGTTGAACTCTCA  
TGTCTCAACCAAACGGATATTTGGTTGCCAAATAAAATCAACTTGAGAGT

301 AATCATGCAGAGCTTTCTGTTACATTAGCGCTGGATGTCCACCAATGCATA  
TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT

351 TGTGGTCGGCTACCGTGCTGGAAATAGCGCATATTTCTTTCATCCTGACA  
ACACCAGCCGATGGCACGACCTTTATCGCGTATAAAGAAAGTAGGACTGT

401 ATCAGGAAGATGCAGAAGCAATCACTCATCTTTTCACTGATGTTCAAAAT  
TAGTCCTTCTACGTCTTCGTTAGTGAGTAGAAAAGTGACTACAAGTTTGA

451 CGATATACATTTCGCCTTTGGTGGTAATTATGATAGACTTGAACAACCTTGC  
GCTATATGTAAGCGGAAACCACCATTAATACTATCTGAACTTGTTGAACG

501 TGGTAATCTGAGAGAAAATATCGAGTTGGGAAATGGTCCACTAGAGGAGG  
ACCATTAGACTCTCTTTTATAGCTCAACCCTTTACCAGGTGATCTCCTCC

551 CTATCTCAGCGCTTTATTATTACAGTACTGGTGGCACTCAGCTTCCAAC  
TATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA

601 CTGGCTCGTTCCTTTATAATTTGCATCCAAATGATTTTCAAGAGCAGCAAG  
GACCGAGCAAGGAAATATTAAACGTAGGTTTACTAAAGTCTTCGTCGTTT

651 ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA  
TAAGGTTATATAACTCCCTCTTTACGCGTGCTCTTAATCCATGTTGGCCT



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**FIGURE 39C (P2)**

701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGGAGA  
CTAGACGTGGTCTAGGATCGCATTAATGTGAACTCTTATCAACCCCTCT

751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTTGCTAGTCCAAT  
GAAAGGTGACGTTAAGTTCTCAGATTGGTTCCTCGGAAACGATCAGGTTA

801 TCAACTGCAAAGACGTAATGGTTCCAAATTCAGTGTGTACGATGTGAGTA  
AGTTGACGTTTCTGCAATTACCAAGGTTTAAGTCACACATGCTACACTCAT

851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGCACCTCCACCA  
ATAATTAGGGATAGTATCGAGAGTACCACATATCTACGCGTGGAGGTGGT

901 TCGTCACAGTTTAAAAAATCCCCTGGAAGAGTTGTGGTGGCTCTGTAGC  
AGCAGTGTCAAATTTTTTAGGGGACCTTCTCAACAGCCACCGAGACATCG

951 TGATGTTTGTATGGATCCTGAGCCCATAGTGCGTATCGTAGGTCGAAATG  
ACTACAAACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTTAC

1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA  
CAGATACACAACACTACAATCCCTACCTTCTAAGGTGTTGCCTTTGCGTTAT

1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT  
GTCAACACCGGTACGTTTACGATTATGTCTACGTTTAGTCGAGACCTGAAA

1101 GAAAAGAGACAATACTATTCGATCTAATGGAAAGTGTTAACTACTTACG  
CTTTTCTCTGTTATGATAAGCTAGATTACCTTTCACAAATTGATGAATGC

1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA  
CCATGTCAGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT

1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC  
TGACTACGGTGGGCGACCGTTTATACCTATTACCTTGGTAGTATTTAGG

1251 CAGATCTAGTCTAGTTTTAGCAGCGACATCAGGGAACAGTGGTACCACAC  
GTCTAGATCAGATCAAAATCGTCGCTGTAGTCCCTTGTACCATGGTGTG

1301 TTACAGTGCAAACCAACATTTATGCCGTTAGTCAAGGTTGGCTTCCTACT  
AATGTCACGTTTGGTTGTAAATACGGCAATCAGTTCCAACCGAAGGATGA

1351 AATAATACACAACCTTTTGTTACAACCATTTGTTGGGCTATATGGTCTGTG  
TTATTATGTGTTGGAAAACAATGTTGGTAACAACCCGATATAACCAGACAC

1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA  
GAACGTTTCGTTTATCACCTGTTTACATACCTATCTCCTGACATCGTCACTTT

0951454\_04400

[illegible][illegible][illegible]

	1970	1971	1972	1973	1974	1975	1976	1977	1978	1979	1980	1981	1982	1983	1984	1985	1986	1987	1988	1989	1990	1991	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	2014	2015	2016	2017	2018	2019	2020	2021	2022	2023	2024	2025	2026	2027	2028	2029	2030	2031	2032	2033	2034	2035	2036	2037	2038	2039	2040	2041	2042	2043	2044	2045	2046	2047	2048	2049	2050	2051	2052	2053	2054	2055	2056	2057	2058	2059	2060	2061	2062	2063	2064	2065	2066	2067	2068	2069	2070	2071	2072	2073	2074	2075	2076	2077	2078	2079	2080	2081	2082	2083	2084	2085	2086	2087	2088	2089	2090	2091	2092	2093	2094	2095	2096	2097	2098	2099	2100	2101	2102	2103	2104	2105	2106	2107	2108	2109	2110	2111	2112	2113	2114	2115	2116	2117	2118	2119	2120	2121	2122	2123	2124	2125	2126	2127	2128	2129	2130	2131	2132	2133	2134	2135	2136	2137	2138	2139	2140	2141	2142	2143	2144	2145	2146	2147	2148	2149	2150	2151	2152	2153	2154	2155	2156	2157	2158	2159	2160	2161	2162	2163	2164	2165	2166	2167	2168	2169	2170	2171	2172	2173	2174	2175	2176	2177	2178	2179	2180	2181	2182	2183	2184	2185	2186	2187	2188	2189	2190	2191	2192	2193	2194	2195	2196	2197	2198	2199	2200	2201	2202	2203	2204	2205	2206	2207	2208	2209	2210	2211	2212	2213	2214	2215	2216	2217	2218	2219	2220	2221	2222	2223	2224	2225	2226	2227	2228	2229	2230	2231	2232	2233	2234	2235	2236	2237	2238	2239	2240	2241	2242	2243	2244	2245	2246	2247	2248	2249	2250	2251	2252	2253	2254	2255	2256	2257	2258	2259	2260	2261	2262	2263	2264	2265	2266	2267	2268	2269	2270	2271	2272	2273	2274	2275	2276	2277	2278	2279	2280	2281	2282	2283	2284	2285	2286	2287	2288	2289	2290	2291	2292	2293	2294	2295	2296	2297	2298	2299	2300	2301	2302	2303	2304	2305	2306	2307	2308	2309	2310	2311	2312	2313	2314	2315	2316	2317	2318	2319	2320	2321	2322	2323	2324	2325	2326	2327	2328	2329	2330	2331	2332	2333	2334	2335	2336	2337	2338	2339	2340	2341	2342	2343	2344	2345	2346	2347	2348	2349	2350	2351	2352	2353	2354	2355	2356	2357	2358	2359	2360	2361	2362	2363	2364	2365	2366	2367	2368	2369	2370	2371	2372	2373	2374	2375	2376	2377	2378	2379	2380	2381	2382	2383	2384	2385	2386	2387	2388	2389	2390	2391	2392	2393	2394	2395	2396	2397	2398	2399	2400	2401	2402	2403	2404	2405	2406	2407	2408	2409	2410	2411	2412	2413	2414	2415	2416	2417	2418	2419	2420	2421	2422	2
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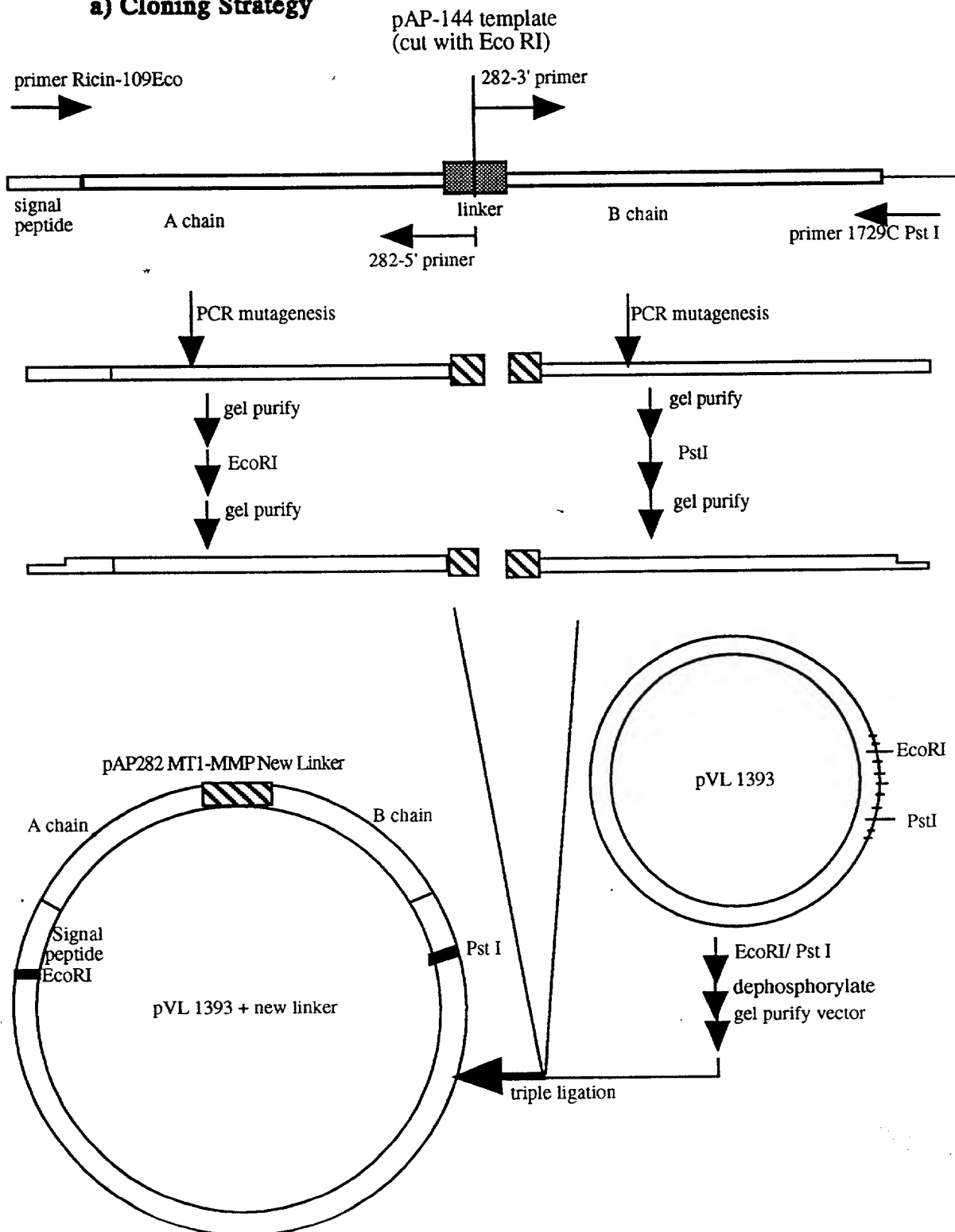
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**FIGURE 39D**

**Figure 39. d) Amino acid sequence Comparison of Mutant Preproricin Linker region of Urokinase-Type Plasminogen Activator to Wild Type**

Wild type ricin linker:           A chain- S L L I R P V V P N F N -B chain  
pAP-280 (uPA) linker:           A chain- K K S P G R V V G G S V-B chain

00440" 6576960

**FIGURE 40A****PCR Mutagenesis of Preproricin Gene to Create A Variant Gene in Baculovirus Transfer Vector, pVL 1393****a) Cloning Strategy**

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## **FIGURE 40B**

### **Sequence of MT-MMP Linker Region**

#### **WT preprocin linker**

primer 282-3'

5' - GCTCCTGGTATTCTTGGCGCTGATGTTTGT -3'

\*\*\*\*\* \* \* \*

-----TCTTTGCTTATAAGGCCA | GTGGTACCAAATTTTAAT-----  
-----AGAAACGAATATTCCGGT | CACCATGGTTTAAAATTA-----  
\* \* \* \* \*

3' - AGCAGTGTCAAAGGGGTTCTTGAGGATCCC -5'

primer 282-5'

1) PCR mutagenesis

2) Ligate with pVL1393

**pAP 282 linker**

**(MT-MMP variant)**

-----CCCCAAGGACTCCTAGGG | GTCCTGGTATTCTTGGC-----  
-----GGGGTTCCTGAGGATCCC | CGAGGACCATAAGAACCG-----

00440" p34.336

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## FIGURE 40C (P1)

Sequence of pAP282 insert

```

      10      20      30      40      50
      |      |      |      |      |
1  GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT
   CTTAAGTACTTTGGCCCTCCTTTATGATAACATTATACCTACATACGTCA

51  GGCAACATGGCTTTGTTTTGGATCCACCTCAGGGTGGTCTTTCACATTAG
   CCGTTGTACCGAAACAAACCTAGGTGGAGTCCCACCAGAAAGTGTATC

101 AGGATAACAACATATTCCCCAAACAATACCCAATTATAAACTTTACCACA
   TCCTATTGTTGTATAAGGGGTTTGTATGGGTTAATATTTGAAATGGTGT

151 GCGGGTGCCACTGTGCAAAGCTACACAACTTTATCAGAGCTGTTCGCGG
   CGCCACGGTGACACGTTTCGATGTGTTTGAAATAGTCTCGACAAGCGCC

201 TCGTTTAACAACCTGGAGCTGATGTGAGACATGATATAACAGTGTTGCCAA
   AGCAAATTGTTGACCTCGACTACACTCTGTACTATATGGTCACAACGGTT

251 ACAGAGTTGGTTTGCCTATAAACCAACGGTTTATTTTAGTTGAACTCTCA
   TGTCTCAACCAACGGATATTTGGTTGCCAAATAAAATCAACTTGAGAGT

301 AATCATGCAGAGCTTTCTGTTACATTAGCGCTGGATGTCACCAATGCATA
   TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT

351 TGTGGTCGGCTACCGTGCTGGAAATAGCGCATATTTCTTTCATCCTGACA
   ACACCAGCCGATGGCACGACCTTTATCGCGTATAAAGAAAGTAGGACTGT

401 ATCAGGAAGATGCAGAAGCAATCACTCATCTTTTCACTGATGTTCAAAAT
   TAGTCCTTCTACGTCTTCGTTAGTGAGTAGAAAAGTGACTACAAGTTTTA

451 CGATATACATTGCGCTTTGGTGGTAATTATGATAGACTTGAACAACCTTGC
   GCTATATGTAAGCGGAAACCACCATTAATACTATCTGAACTTGTTGAACG

501 TGGTAATCTGAGAGAAAATATCGAGTTGGGAAATGGTCCACTAGAGGAGG
   ACCATTAGACTCTCTTTTATAGCTCAACCCTTTACCAGGTGATCTCCTCC

551 CTATCTCAGCGCTTTATTATTACAGTACTGGTGGCACTCAGCTTCCAAC
   TATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA

601 CTGGCTCGTTCCTTTATAATTTGCATCCAAATGATTTTCAAGAGCAGCAAG
   GACCGAGCAAGGAAATATTAAACGTAGGTTTACTAAAGTCTTCGTCGTTTC

651 ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA
   TAAGGTTATATAACTCCCTCTTTACGCGTGCTCTTAATCCATGTTGGCCT
```

095453.04400

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**FIGURE 40C (P2)**

701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGGAGA  
CTAGACGTGGTCTAGGATCGCATTAAATGTGAACTCTTATCAACCCCTCT

751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTTGCTAGTCCAAT  
GAAAGGTGACGTTAAGTTCTCAGATTGGTTCCTCGGAAACGATCAGGTTA

801 TCAACTGCAAAGACGTAATGGTTCCAAATTCAGTGTGTACGATGTGAGTA  
AGTTGACGTTTCTGCATTACCAAGGTTTAAAGTCACACATGCTACACTCAT

851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGCACCTCCACCA  
ATAATTAGGGATAGTATCGAGAGTACCACATATCTACGCGTGGAGGTGGT

901 TCGTCACAGTTTCCCCAAGGACTCCTAGGGGCTCCTGGTATTCTTGGCGC  
AGCAGTGTCAAAGGGGTTCTGAGGATCCCCGAGGACCATAAGAACCGCG

951 TGATGTTTGTATGGATCCTGAGCCCATAGTGCGTATCGTAGGTCGAAATG  
ACTACAAACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTTAC

1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA  
CAGATACACAACCTACAATCCCTACCTTCTAAGGTGTTGCCTTTGCGTTAT

1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT  
GTCAACACCGGTACGTTTACGATTATGTCTACGTTTAGTCGAGACCTGAAA

1101 GAAAAGAGACAATACTATTTCGATCTAATGGAAAGTGTTTAACTACTTACG  
CTTTTCTCTGTTATGATAAGCTAGATTACCTTTCACAAATTGATGAATGC

1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA  
CCATGTCAGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT

1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC  
TGACTACGGTGGGCGACCGTTTATACCCTATTACCTTGGTAGTATTTAGG

1251 CAGATCTAGTCTAGTTTTAGCAGCGACATCAGGGAACAGTGGTACCACAC  
GTCTAGATCAGATCAAAATCGTCGCTGTAGTCCCTTGTCACCATGGTGTG

1301 TTACAGTGCAAACCAACATTTATGCCGTTAGTCAAGGTTGGCTTCCTACT  
AATGTCACGTTTGGTTGTAAATACGGCAATCAGTTCCAACCGAAGGATGA

1351 AATAATACACAACCTTTTGTACCAACCATTTGTTGGGCTATATGGTCTGTG  
TTATTATGTGTTGGAAAACAATGTTGGTAACAACCCGATATACCAGACAC

1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA  
GAACGTTTCGTTTATCACCTGTTTCATACCTATCTCCTGACATCGTCACTTT

0055151 "041400

**FIGURE 40C (P3)**

1451 AGGCTGAACAACAGTGGGCTCTTTATGCAGATGGTTCAATACGTCCTCAG  
TCCGACTTGTTGTACCCCGAGAAATACGTCTACCAAGTTATGCAGGAGTC

1501 CA<sup>^</sup>AAACCGAGATAATTGCCTTACAAGTGATTCTAATATACGGGAAACAGT  
GTTTTGGCTCTATTAACGGAATGTTCACTAAGATTATATGCCCTTTGTCA

1551 TGTTAAGATCCTCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT  
ACAATTCTAGGAGAGAACACCGGGACGTAGGAGACCGGTTGCTACCTACA

1601 TCAAGAATGATGGAACCATTTTAAATTTGTATAGTGGATTGGTGTTAGAT  
AGTTCTTACTACCTTGGTAAAATTTAAACATATCACCTAACCACAATCTA

1651 GTGAGGGCGATCGGATCCGAGCCTTAAACAAATCATTCTTTACCCTCTCCA  
CACTCCGCTAGCCTAGGCTCGGAATTTGTTTAGTAAGAAATGGGAGAGGT

1701 TGGTGACCCAAACCAAATATGGTTACCATTTATTTGATAGACAGATTACT  
ACCACTGGGTTTGGTTTATACCAATGGTAATAAACTATCTGTCTAATGA

1751 CTCTTGCAGTGTGTGTGTCTCTGCCATGAAAATAGATGGCTTAAATAAAAA  
GAGAACGTCACACACACAGGACGGTACTTTTATCTACCGAATTTATTTTTT

1801 GGACATTGTAAATTTTGTAACTGAAAGGACAGCAAGTTATATCGAATTCC  
CCTGTAACATTTAAACATTGACTTTCCTGTGCTTCAATATAGCTTAAGG

1851 TGCAG  
ACGTC

Total number of bases is: 1855.

Sequence name: PAP282



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**FIGURE 40D**

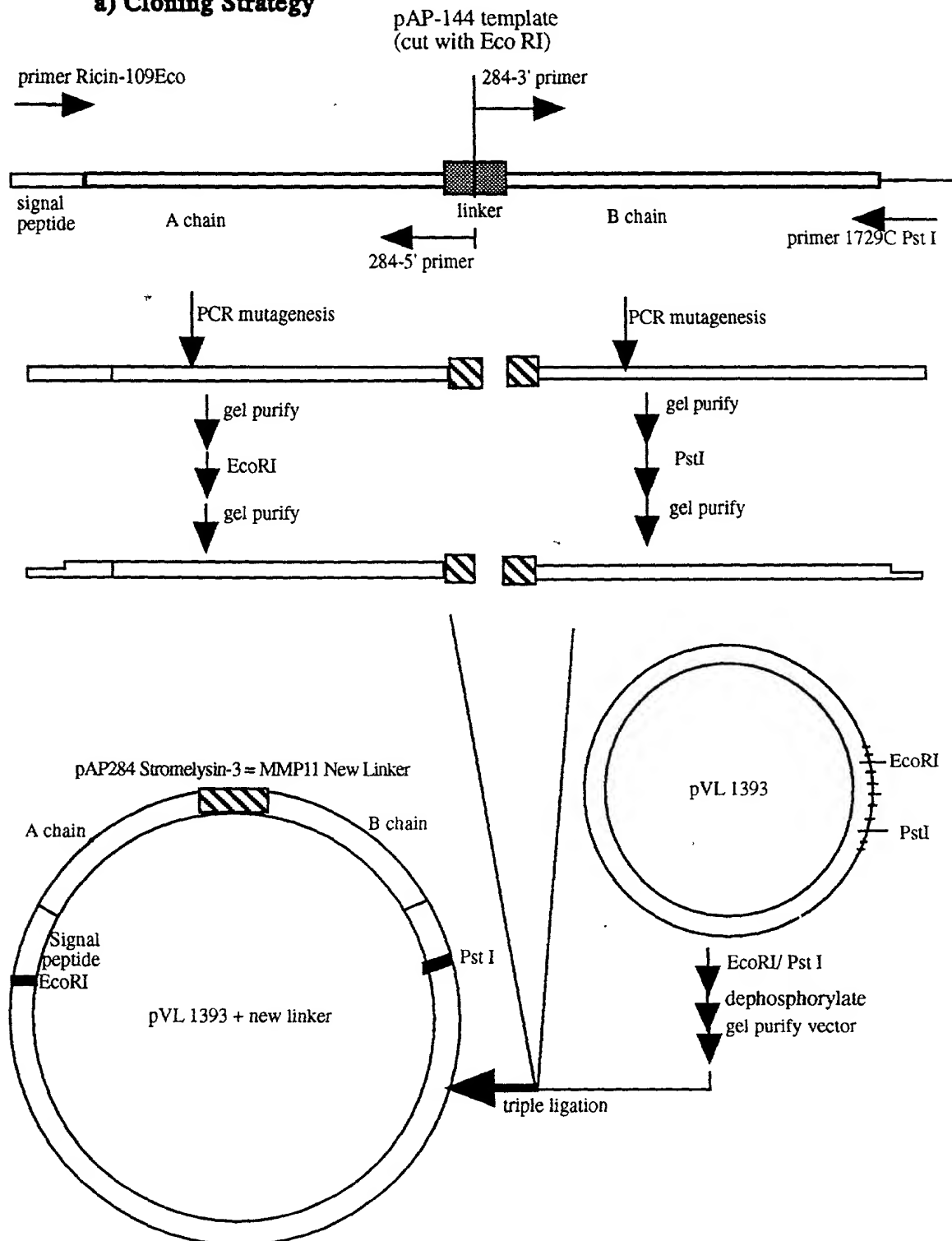
**Amino acid sequence Comparison of Mutant Preproricin Linker  
region of MT-MMP to Wild Type**

Wild type ricin linker:	A chain- S L L I R P V V P N F N -B chain
pAP-282 (MT-MMP) linker:	A chain- P Q G L L G A P G I L G-B chain

00440" EST 5560

**FIGURE 41A**

**PCR Mutagenesis of Preproricin Gene to Create A Variant Gene in Baculovirus Transfer Vector, pVL 1393**

**a) Cloning Strategy**

## FIGURE 41B

### Sequence of MMP-11 (Stromelysin-3) Linker Region

#### WT preprocin linker

primer 284-3'  
 5' - ATGGGAAGAGGCCCATGCTCGTTTAGTTTCATGTCGAAAGAGCCTCACACTGCTGATGTTTGTATGGAT-3'  
 -----TCTTTTGCTTATAAGGCCA | GTGGTACCAAAATTTTAAT-----  
 -----AGAAACGGAATATCCGGT | CACCATGGTTTAAAAATTA-----

3'-GGTGGTAGCAGTGTCAAAGTGCCTGGGGCTCCCAAATTTCTCACCCTAAAAATACTTAGACTGCAG -5'  
 primer 284-5'

1) PCR mutagenesis

2) Ligate with pVL1393

#### pAP 284 linker

(MMP-11 variant)

---CACGGCCCCGAGGGTTTAAGAGTGGGATTTTATGAATCTGACGTC | ATGGGAAGAGGCCCATGCTCGTTTAGTTTCATGTCGAAAGAGCCTCACACT---  
 ---GTGCGGGGGCTCCCAAATTTCTCACCCTAAAAATACTTAGACTGCAG | TACCCCTTCTCCGGTACGAGCAAAATCAAGTACAGCAACTCGGAGTGTGA---

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## FIGURE 41C (P1)

Sequence of pAP284 insert

```

      10      20      30      40      50
      |      |      |      |      |
1  GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT
   CTTAAGTACTTTGGCCCTCCTTTATGATAACATTATACCTACATACGTCA

51  GGCAACATGGCTTTGTTTTGGATCCACCTCAGGGTGGTCTTTCACATTAG
   CCGTTGTACCGAAACAAACCTAGGTGGAGTCCCACCAGAAAGTGTATC

101 AGGATAACAACATATTCCCCAAACAATACCCAATTATAAACTTTACCACA
   TCCTATTGTTGTATAAGGGGTTTGTATGGGTTAATATTTGAAATGGTGT

151 GCGGGTGCCACTGTGCAAAGCTACACAACTTTATCAGAGCTGTTGCGGG
   CGCCCACGGTGACACGTTTCGATGTGTTTGAAATAGTCTCGACAAGCGCC

201 TCGTTTAACAACCTGGAGCTGATGTGAGACATGATATACCACTGTTGCCAA
   AGCAAATTGTTGACCTCGACTACACTCTGTACTATATGGTCACAACGGTT

251 ACAGAGTTGGTTTGCCTATAAACCAACGGTTTATTTTAGTTGAACTCTCA
   TGTCTCAACCAAACGGATATTTGGTTGCCAAATAAAATCAACTTGAGAGT

301 AATCATGCAGAGCTTCTGTTACATTAGCGCTGGATGTCACCAATGCATA
   TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT

351 TGTGGTCGGCTACCGTGCTGGAAATAGCGCATATTTCTTTCATCCTGACA
   ACACCAGCCGATGGCACGACCTTTATCGCGTATAAAGAAAGTAGGACTGT

401 ATCAGGAAGATGCAGAAGCAATCACTCATCTTTTCACTGATGTTCAAAAT
   TAGTCCTTCTACGTCTTCGTTAGTGAGTAGAAAAGTGAAGTCAAGTTTGA

451 CGATATACATTTCGCCTTTGGTGGTAATTATGATAGACTTGAACAACTTGC
   GCTATATGTAAGCGGAAACCACCATTAATACTATCTGAACCTGTTGAACG

501 TGGTAATCTGAGAGAAAATATCGAGTTGGGAAATGGTCCACTAGAGGAGG
   ACCATTAGACTCTCTTTTATAGCTCAACCCTTACCAGGTGATCTCCTCC

551 CTATCTCAGCGCTTTATTATTACAGTACTGGTGGCACTCAGCTTCCAAC
   TATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA

601 CTGGCTCGTTCCTTTATAATTTGCATCCAAATGATTTTCAAGAGCAGCAAG
   GACCGAGCAAGGAAATATTAAACGTAGGTTTACTAAAGTCTTCGTCGTTT

651 ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA
   TAAGGTTATATAACTCCCTCTTTACGCGTGCTCTTAATCCATGTTGGCCT
```

095410151550

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**FIGURE 41C (P2)**

701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGGAGA  
CTAGACGTGGTCTAGGATCGCATTAAATGTGAACTCTTATCAACCCCCTCT

751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTTGCTAGTCCAAT  
GAAAGGTGACGTTAAGTTCTCAGATTGGTTCCTCGGAAACGATCAGGTTA

801 TCAACTGCAAAGACGTAATGGTTCCAAATTCAGTGTGTACGATGTGAGTA  
AGTTGACGTTTCTGCATTACCAAGGTTTAAAGTCACACATGCTACACTCAT

851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGCACCTCCACCA  
ATAATTAGGGATAGTATCGAGAGTACCACATATCTACGCGTGGAGGTGGT

901 TCGTCACAGTTT  
AGCAGTGTCAAA

Linker Sequence:

CACGGCCCCGAGGGTTTAAGAGTGGGATTTTATGAATCTGACGTCATGGG  
GTGCCGGGGCTCCCAAATTCTCACCCCTAAAATACTTAGACTGCAGTACCC

AAGAGGCCATGCTCGTTTAGTTTCATGTGGAAGAGCCTCACACT  
TTCTCCGGTACGAGCAAATCAAGTACAGCAACTCGGAGTGTGA

949 GC  
CG

951 TGATGTTTGATGGATCCTGAGCCCATAGTGCGTATCGTAGGTCGAAATG  
ACTACAAACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTTAC

1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA  
CAGATACACAACACTACAATCCCTACCTTCTAAGGTGTTGCCTTTGCGTTAT

1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT  
GTCAACACCGGTACGTTTCAGATTATGTCTACGTTTAGTCGAGACCTGAAA

1101 GAAAAGAGACAATACTATTTCGATCTAATGGAAAGTGTTTAACTACTTACG  
CTTTTCTCTGTTATGATAAGCTAGATTACCTTTCACAAATTGATGAATGC

1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA  
CCATGTCAGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT

1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC  
TGACTACGGTGGGCGACCGTTTATACCCTATTACCTTGGTAGTATTTAGG

1251 CAGATCTAGTCTAGTTTTAGCAGCGACATCAGGGAACAGTGGTACCACAC  
GTCTAGATCAGATCAAATCGTCGCTGTAGTCCCTTGTCAACCATGGTGTG



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**FIGURE 41D**

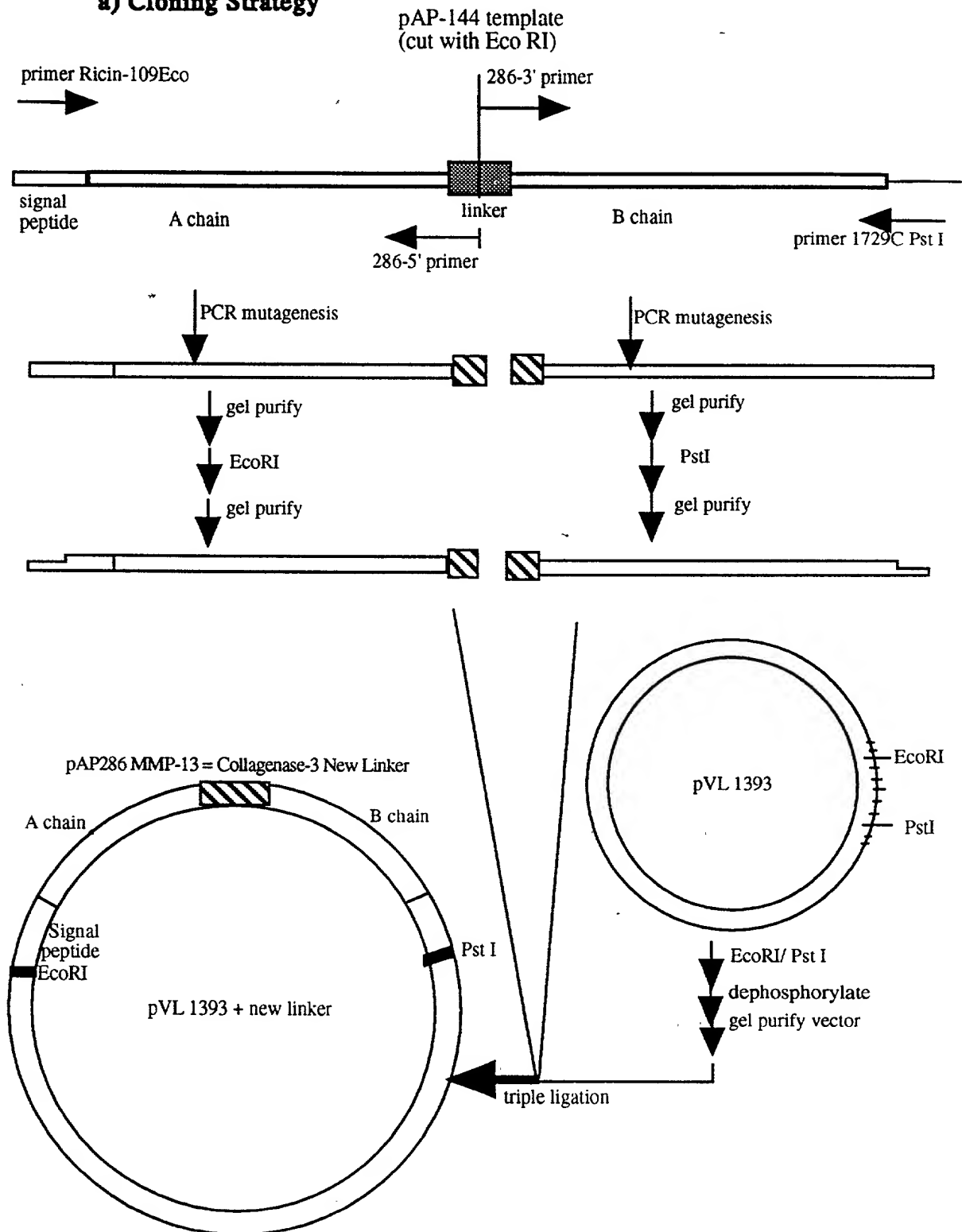
**Amino acid sequence Comparison of Mutant Preproricin Linker  
region of MMP-11 (Stromelysin-3) to Wild Type**

Wild type ricin linker:           A chain- S L L I R P V V P N F N -B chain

pAP-284 (MMP-11) linker:

A chain- H G P E G L R V G F Y E S D V M G R G H A R L V H V E E P H T -B chain

004440 151550

**FIGURE 42A****PCR Mutagenesis of Preproricin Gene to Create A Variant Gene in Baculovirus Transfer Vector, pVL 1393****a) Cloning Strategy**



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## FIGURE 42B

Sequence of MMP-13 = Collagenase-3 Linker Region

### WT preprocin linker

primer 286-3'  
5' - GGTCAACGAGGCATTGTCGCTGATGTTTGT -3'  
\*\*\*\*\* \* \*\*\*\*\* \*\*\*  
-----TCTTTGCTTATAAGGCCA|GTGGTACCAAATTTTAAT-----  
-----AGAAACGAATATTCCGGT|CACCATGGTTTAAATTA-----  
\*\*\*\*\* \*\*\*\*\* \*  
3' -AGCAGTGTCAAACCTGGAGTCCCCGAACGA -5'  
primer 286-5'

1) PCR mutagenesis

2) Ligate with pVL1393

### pAP 286 linker (MMP-13 variant)

-----GGACCTCAGGGGCTTGCT|GGTCAACGAGGCATTGTC-----  
-----CCTGGAGTCCCCGAACGA|CCAGTTGCTCCGTAAACAG-----

00440" 63650

201/254

**FIGURE 42C (P1)**

Sequence of pAP286 insert

```

      10      20      30      40      50
      |      |      |      |      |
1  GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT
   CTTAAGTACTTTGGCCCTCCTTTATGATAACATTATACCTACATACGTCA

51  GGCAACATGGCTTTGTTTTGGATCCACCTCAGGGTGGTCTTTCACATTAG
   CCGTTGTACCGAAACAAACCTAGGTGGAGTCCCACCAGAAAGTGTAAATC

101 AGGATAACAACATATTCCCCAAACAATACCCAATTATAAACTTTACCACA
   TCCTATTGTTGTATAAGGGGTTTGTATGGGTTAATATTTGAAATGGTGT

151 GCGGGTGCCACTGTGCAAAGCTACACAACTTTATCAGAGCTGTTGCGGG
   CGCCACGGTGACACGTTTCGATGTGTTTGAAATAGTCTCGACAAGCGCC

201 TCGTTTAACAACTGGAGCTGATGTGAGACATGATATAACAGTGTGCGCAA
   AGCAAATTGTTGACCTCGACTACACTCTGTACTATATGGTCACAACGGTT

251 ACAGAGTTGGTTTGCCTATAAACCAACGGTTTATTTTAGTTGAACTCTCA
   TGTCTCAACCAAACGGATATTTGGTTGCCAAATAAAATCAACTTGAGAGT

301 AATCATGCAGAGCTTCTGTGTACATTAGCGCTGGATGTCACCAATGCATA
   TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT

351 TGTGGTCGGCTACCGTGCTGGAAATAGCGCATATTTCTTTCATCCTGACA
   ACACCAGCCGATGGCACGACCTTTATCGCGTATAAAGAAAGTAGGACTGT

401 ATCAGGAAGATGCAGAAGCAATCACTCATCTTTTCACTGATGTTCAAAT
   TAGTCCTTCTACGTCTTCGTTAGTGAGTAGAAAAGTGACTACAAGTTTTA

451 CGATATACATTTCGCCTTTGGTGGTAATTATGATAGACTTGAACAACTTGC
   GCTATATGTAAGCGGAAACCACCATTAACTATCTGAAGTTGTTGAACG

501 TGGTAATCTGAGAGAAAATATCGAGTTGGGAAATGGTCCACTAGAGGAGG
   ACCATTAGACTCTCTTTTATAGCTCAACCCTTACCAGGTGATCTCCTCC

551 CTATCTCAGCGCTTTATTATTACAGTACTGGTGGCACTCAGCTTCCAAC
   TATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA

601 CTGGCTCGTTTCCTTTATAATTTGCATCCAAATGATTTCAGAAGCAGCAAG
   GACCGAGCAAGGAAATATTAAACGTAGGTTTACTAAAGTCTTCGTCGTTT

651 ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA
   TAAGGTTATATAACTCCCTCTTTACGCGTGCTCTTAATCCATGTTGGCCT

701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGGAGA
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**FIGURE 42C (P2)**

CTAGACGTGGTCTAGGATCGCATTAAATGTGAACTCTTATCAACCCCCTCT

751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTTGCTAGTCCAAT  
GAAAGGTGACGTTAAGTTCTCAGATTGGTTCCTCGGAAACGATCAGGTTA

801 TCAACTGCAAAGACGTAATGGTTCCAAATTCAGTGTGTACGATGTGAGTA  
AGTTGACGTTTCTGCATTACCAAGGTTTAAGTCACACATGCTACACTCAT

851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGCACCTCCACCA  
ATAATTAGGGATAGTATCGAGAGTACCACATATCTACGCGTGGAGGTGGT

901 TCGTCACAGTTTGGACCTCAGGGGCTTGCTGGTCAACGAGGCATTGTTCG  
AGCAGTGTCAAACCTGGAGTCCCCGAACGACCAGTTGCTCCGTAACAGCG

951 TGATGTTTGTATGGATCCTGAGCCCATAGTGCGTATCGTAGGTCGAAATG  
ACTACAAACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTTAC

1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA  
CAGATACACAACCTACAATCCCTACCTTCTAAGGTGTTGCCTTTGCGTTAT

1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT  
GTCAACACCGGTACGTTTACGATTATGTCTACGTTTGTAGTCGAGACCTGAAA

1101 GAAAAGAGACAATACTATTTCGATCTAATGGAAAGTGTTTAACTACTTACG  
CTTTTCTCTGTTATGATAAGCTAGATTACCTTTCACAAATTGATGAATGC

1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA  
CCATGTACAGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT

1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC  
TGACTACGGTGGGCGACCGTTTATACCCTATTACCTTGGTAGTATTTAGG

1251 CAGATCTAGTCTAGTTTTAGCAGCGACATCAGGGAACAGTGGTACCACAC  
GTCTAGATCAGATCAAAATCGTCGCTGTAGTCCCTTGTCAACCATGGTGTG

1301 TTACAGTGCAAACCAACATTTATGCCGTTAGTCAAGGTTGGCTTCCTACT  
AATGTCACGTTTGGTTGTAAATACGGCAATCAGTTCCAACCGAAGGATGA

1351 AATAATACACAACCTTTTGTTACAACCATTTGTTGGGCTATATGGTCTGTG  
TTATTATGTGTTGGAAAACAATGTTGGTAACAACCCGATATACCAGACAC

1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA  
GAACGTTTCGTTTATCACCTGTTTCATACCTATCTCCTGACATCGTCACTTT

1451 AGGCTGAACAACAGTGGGCTCTTTATGCAGATGGTTCAATACGTCCTCAG  
TCCGACTTGTTGTCACCCGAGAAATACGTCCTACCAAGTTATGCAGGAGTC

1501 CAAAACCGAGATAATTGCCTTACAAGTGATTCTAATATACGGGAAACAGT

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**FIGURE 42C (P3)**

GTTTTGGCTCTATTAACGGAATGTTCACTAAGATTATATGCCCTTTGTCA  
1551 TGTAAAGATCCTCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT  
ACAATTCTAGGAGAGAACACCGGGACGTAGGAGACCGGTGCTACCTACA  
1601 TCAAGAATGATGGAACCATTTTAAATTTGTATAGTGGATTGGTGTTAGAT  
AGTTCTTACTACCTTGGTAAAATTTAAACATATCACCTAACCACAATCTA  
1651 GTGAGGCGATCGGATCCGAGCCTTAAACAAATCATTCTTTACCCTCTCCA  
CACTCCGCTAGCCTAGGCTCGGAATTTGTTTAGTAAGAAATGGGAGAGGT  
1701 TGGTGACCCAAACCAAATATGGTTACCATTATTTTGATAGACAGATTACT  
ACCACTGGGTTTGGTTTATACCAATGGTAATAAAACTATCTGTCTAATGA  
1751 CTCTTGCAAGTGTGTGTGTCCTGCCATGAAAATAGATGGCTTAAATAAAAA  
GAGAACGTCACACACACAGGACGGTACTTTTATCTACCGAATTTATTTTT  
1801 GGACATTGTAAATTTTGTAACTGAAAGGACAGCAAGTTATATCGAATTCC  
CCTGTAAACATTTAAACATTGACTTTCCTGTCGTTCAATATAGCTTAAGG  
1851 TGCAG  
ACGTC

0959151 041400

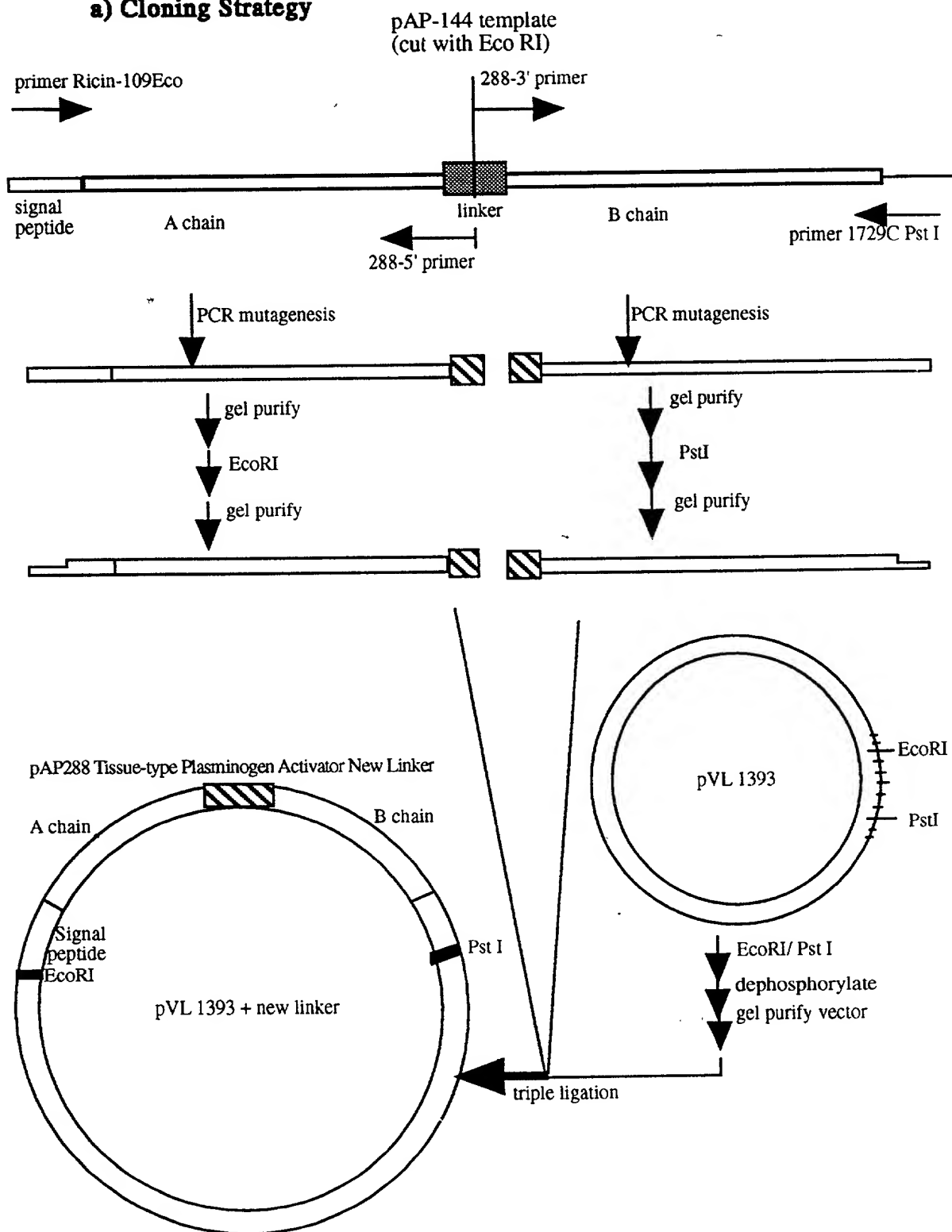
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**FIGURE 42D**

**Amino acid sequence Comparison of Mutant Preproricin Linker  
region of MMP-13 (Collagenase-3) to Wild Type**

Wild type ricin linker:      A chain- S L L I R P V V P N F N -B chain  
pAP-286 (MMP-13) linker:      A chain- G P Q G L A G Q R G I V -B chain

004E40" F5F5560

**FIGURE 43A****PCR Mutagenesis of Preproricin Gene to Create A Variant Gene in Baculovirus Transfer Vector, pVL 1393****a) Cloning Strategy**

**FIGURE 43B****Sequence of Tissue-type Plasminogen Activator (tPA) Linker Region****WT preprocin linker**

primer 288-3'  
 5' - GGTCGTAAAGCTCTTGAAGCTGATGTTTGT -3'  
 \*\*\*\*\* \*\* \* \*

-----TCTTTGCTTATAAGGCCA|GTGGTACCAAATTTTAAT-----  
 -----AGAAACGAATATTCCGGT|CACCATGGTTTAAAATTA-----  
 \*\*\*\*\* \*\*\*\*\*

3' -AGCAGTGTCAAACCGCCTAGACCCGTTTCC -5'  
 primer 288-5'

1) PCR mutagenesis

2) Ligate with pVL1393

**pAP 288 linker  
(tPA variant)**

-----GGCGGATCTGGGCAAAGG|GGTCGTAAAGCTCTTGAA-----  
 -----CCGCCTAGACCCGTTTCC|CCAGCATTTTCGAGAACTT-----

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## **FIGURE 43C (P1)**

Sequence of pAP288 insert

```

          10          20          30          40          50
          |          |          |          |          |
1  GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT
   CTTAAGTACTTTGGCCCTCCTTTATGATAACATTATACCTACATACGTCA

51  GGCAACATGGCTTTGTTTTGGATCCACCTCAGGGTGGTCTTTCACATTAG
   CCGTTGTACCGAAACAAACCTAGGTGGAGTCCCACCAGAAAGTGTAATC

101 AGGATAACAACATATTCCCCAAACAATACCCAATTATAAACTTTACCACA
   TCCTATTTGTTGTATAAGGGGTTTGTATGGGTAAATATTTGAAATGGTGT

151 GCGGGTGCCACTGTGCAAAGCTACACAACTTTATCAGAGCTGTTGCGGG
   CGCCACGGTGACACGTTTCGATGTGTTTGAAATAGTCTCGACAAGCGCC

201 TCGTTTAACAACCTGGAGCTGATGTGAGACATGATATAACAGTGTTGCCAA
   AGCAAATTGTTGACCTCGACTACACTCTGTACTATATGGTCACAACGGTT

251 ACAGAGTTGGTTTGCCTATAAACCAACGGTTTATTTTAGTTGAACTCTCA
   TGTCTCAACCAAACGGATATTTGGTTGCCAAATAAAATCAACTTGAGAGT

301 AATCATGCAGAGCTTCTGTGTACATTAGCGCTGGATGTCACCAATGCATA
   TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT

351 TGTGGTCGGCTACCGTGCTGGAAATAGCGCATATTTCTTTCATCCTGACA
   ACACCAGCCGATGGCACGACCTTTATCGCGTATAAAGAAAGTAGGACTGT

401 ATCAGGAAGATGCAGAAGCAATCACTCATCTTTTCACTGATGTTCAAAT
   TAGTCCTTCTACGTCTTCGTTAGTGAGTAGAAAAGTGACTACAAGTTTGA

451 CGATATACATTTCGCCTTTGGTGGTAATTATGATAGACTTGAACAACCTGC
   GCTATATGTAAGCGGAAACCACCATTAATACTATCTGAACCTGTTGAACG

501 TGGTAATCTGAGAGAAAATATCGAGTTGGGAAATGGTCCACTAGAGGAGG
   ACCATTAGACTCTCTTTTATAGCTCAACCCTTACCAGGTGATCTCCTCC

551 CTATCTCAGCGCTTTATTATTACAGTACTGGTGGCACTCAGCTTCCAAC
   TATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA

601 CTGGCTCGTTTCCTTTATAATTTGCATCCAAATGATTTCAGAAGCAGCAAG
   GACCGAGCAAGGAAATATTAAACGTAGGTTTACTAAAGTCTTCGTCGTTG

651 ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA
   TAAGGTTATATAACTCCCTCTTTACGCGTGCTCTTAATCCATGTTGGCCT

701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGGAGA
```

004400 " 654560



**FIGURE 43C (P2)**

CTAGACGTGGTCTAGGATCGCATTAAATGTGAACCTCTTATCAACCCCTCT

751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTTGCTAGTCCAAT  
GAAAGGTGACGTTAAGTTCTCAGATTGGTTCCTCGGAAACGATCAGGTTA

801 TCAACTGCAAAGACGTAATGGTTCCAAATTCAGTGTGTACGATGTGAGTA  
AGTTGACGTTTCTGCATTACCAAGGTTTAAGTCACACATGCTACACTCAT

851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGCACCTCCACCA  
ATAATTAGGGATAGTATCGAGAGTACCACATATCTACGCGTGGAGGTGGT

901 TCGTCACAGTTTGGCGGATCTGGGCAAAGGGGTCGTAAAGCTCTTGAAGC  
AGCAGTGTCAAACCGCCTAGACCCGTTTCCCCAGCATTTCGAGAACTTCG

951 TGATGTTTGTATGGATCCTGAGCCCATAGTGC GTATCGTAGGTCGAAATG  
ACTACAAACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTTAC

1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA  
CAGATACACAAC TACAATCCCTACCTTCTAAGGTGTTGCCTTTGCGTTAT

1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT  
GTCAACACCGGTACGTT CAGATTATGTCTACGTTTAGTCGAGACCTGAAA

1101 GAAAAGAGACAATACTATTTCGATCTAATGGAAAGTGTTTAACTACTTACG  
CTTTTCTCTGTTATGATAAGCTAGATTACCTTTCACAAATTGATGAATGC

1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA  
CCATGT CAGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT

1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC  
TGACTACGGTGGGCGACCGTTTATACCCTATTACCTTGGTAGTATTTAGG

1251 CAGATCTAGTCTAGTTTTAGCAGCGACATCAGGGAACAGTGGTACCACAC  
GTCTAGATCAGATCAAATCGTCGCTGTAGTCCCTTGT CACCATGGTGTG

1301 TTACAGTGCAAACCAACATTTATGCCGTTAGTCAAGGTTGGCTTCCTACT  
AATGTCACGTTTGGTTGTAAATACGGCAATCAGTTCCAACCGAAGGATGA

1351 AATAATACACAACCTTTTGTTACAACCATTGTTGGGCTATATGGTCTGTG  
TTATTATGTGTTGGAAAACAATGTTGGTAACAACCCGATATACCAGACAC

1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA  
GAACGTTTCGTTTATCACCTGTT CATACTATCTCCTGACATCGTCACTTT

1451 AGGCTGAACAACAGTGGGCTCTTTATGCAGATGGTTCAATACGTCCTCAG  
TCCGACTTGTTGTCACCCGAGAAATACGTCTACCAAGTTATGCAGGAGTC

1501 CAAAACCGAGATAATTGCCTTACAAGTGATTCTAATATACGGGAACAGT

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**FIGURE 43C (P3)**

GT TTTGGCTCTATTAACGGAATGTTCACTAAGATTATATGCCCTTTGTCA  
1551 TGTAAAGATCCTCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT  
ACAATTCTAGGAGAGAACACCGGGACGTAGGAGACCGGTTGCTACCTACA  
1601 TCAAGAATGATGGAACCATTTTAAATTTGTATAGTGGATTGGTGTAGAT  
AGTTCTTACTACCTTGGTAAAATTTAAACATATCACCTAACCACAATCTA  
1651 GTGAGGCGATCGGATCCGAGCCTTAAACAAATCATTCTTTACCCCTCTCCA  
CACTCCGCTAGCCTAGGCTCGGAATTTGTTTAGTAAGAAATGGGAGAGGT  
1701 TGGTGAÇCCAAACCAAATATGGTTACCATTATTTTGATAGACAGATTACT  
ACCACTGGGTTTGGTTTATACCAATGGTAATAAACTATCTGTCTAATGA  
1751 CTCTTGCAGTGTGTGTGTCCTGCCATGAAAATAGATGGCTTAAATAAAAA  
GAGAACGTCACACACACAGGACGGTACTTTTATCTACCGAATTTATTTTT  
1801 GGACATTGTAAATTTTGTAAGTAAAGGACAGCAAGTTATATCGAATTCC  
CCTGTAACATTTAAACATTGACTTTCCTGTCGTTCAATATAGCTTAAGG  
1851 TGCAG  
ACGTC

Total number of bases is: 1855.

Sequence name: PAP288

00440"FF56

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**FIGURE 43D**

**Amino acid sequence Comparison of Mutant Preproricin Linker  
region of Tissue-type Plasminogen Activator (tPA) to Wild Type**

Wild type ricin linker:           A chain- S L L I R P V V P N F N -B chain

pAP-288 (tPA) linker:           A chain- G G S G Q R G R K A L E-B chain

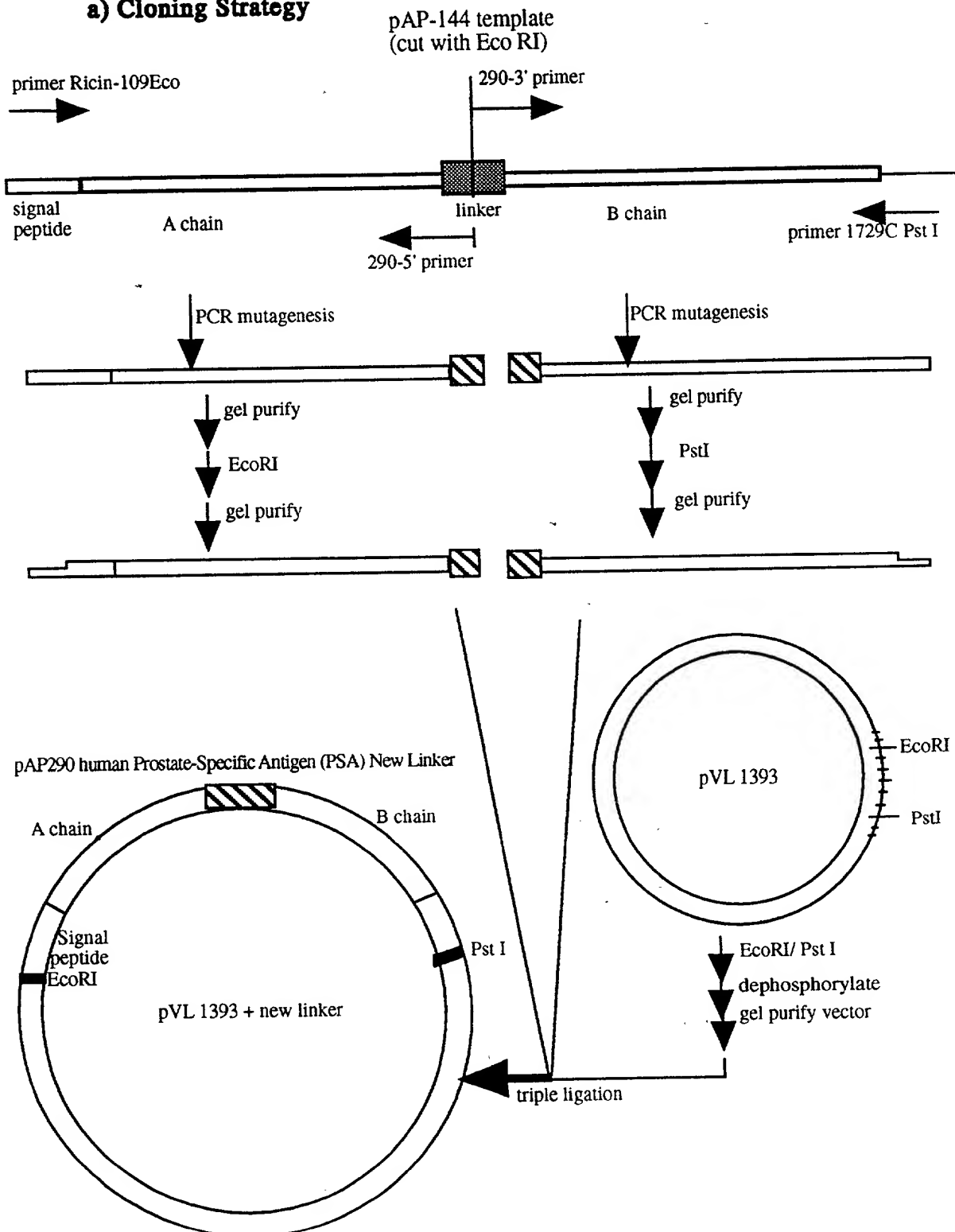
00440"04400

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# FIGURE 44A

**PCR Mutagenesis of Preproricin Gene to Create A Variant Gene in Baculovirus Transfer Vector, pVL 1393**

## a) Cloning Strategy



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## **FIGURE 44B**

### **Sequence of human Prostate-Specific Antigen (PSA) Linker Region**

#### **WT preprocin linker**

primer 290-3'

5' - TCTTCCGATATTTTAAATGCTGATGTTTGT -3'  
\*\*\*\*\* \*

-----TCTTTGCTTATAAGGCCA|GTGGTACCAAATTTTAAT-----  
-----AGAAACGAATATTCCGGT|CACCATGGTTTAAATTA-----  
\* \*\*\*\*\* \*

3' -AGCAGTGTCAAAAGAAACAGTCGAGAAGAG -5'

primer 290-5'

1) PCR mutagenesis

2) Ligate with pVL1393

#### **pAP 290 linker**

**(PSA variant)**

-----TCTTTGTCAGCTCTTCTC|TCTTCCGATATTTTAAAT-----  
-----AGAAACAGTCGAGAAGAG|AGAAGGCTATAAAAATTA-----

09544400 "TCTTCCGATATTTTAAATGCTGATGTTTGT -3'

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**FIGURE 44C (P1)**

Sequence of pAP290 insert

```

      10      20      30      40      50
      |      |      |      |      |
1  GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT
   CTTAAGTACTTTGGCCCTCCTTTATGATAACATTATACCTACATACGTCA

51  GGCAACATGGCTTTGTTTTGGATCCACCTCAGGGTGGTCTTTCACATTAG
   CCGTTGTACCGAAACAAACCTAGGTGGAGTCCCACCAGAAAGTGTAATC

101 AGGATAACAACATATTCCCCAAACAATACCCAATTATAAACTTTACCACA
   TCCTATTGTTGTATAAGGGGTTTGTATGGGTTAATATTTGAAATGGTGT

151 GCGGGTGCCACTGTGCAAAGCTACACAACTTTATCAGAGCTGTTGCGGG
   CGCCCAACGGTGACACGTTTCGATGTGTTTGAAATAGTCTCGACAAGCGCC

201 TCGTTTAACAACCTGGAGCTGATGTGAGACATGATATAACAGTGTGCCAA
   AGCAAATTGTTGACCTCGACTACACTCTGTACTATATGGTCACAACGGTT

251 ACAGAGTTGGTTTGCCTATAAACCAACGGTTTATTTTAGTTGAACTCTCA
   TGTCTCAACCAAACGGATATTTGGTTGCCAAATAAAATCAACTTGAGAGT

301 AATCATGCAGAGCTTTCTGTTACATTAGCGCTGGATGTCACCAATGCATA
   TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT

351 TGTGGTCGGCTACCGTGCTGGAAATAGCGCATATTTCTTTCATCCTGACA
   ACACCAGCCGATGGCACGACCTTTATCGCGTATAAAGAAAGTAGGACTGT

401 ATCAGGAAGATGCAGAAGCAATCACTCATCTTTTCACTGATGTTCAAAT
   TAGTCCTTCTACGTCTTCGTTAGTGAGTAGAAAAGTGACTACAAGTTTTA

451 CGATATACATTTCGCCTTTGGTGGTAATTATGATAGACTTGAACAACTTGC
   GCTATATGTAAGCGGAAACCACCATTAATACTATCTGAACTTGTTGAACG

501 TGGTAATCTGAGAGAAATATCGAGTTGGGAAATGGTCCACTAGAGGAGG
   ACCATTAGACTCTCTTTTATAGCTCAACCCTTACCAGGTGATCTCCTCC

551 CTATCTCAGCGCTTTATTATTACAGTACTGGTGGCACTCAGCTTCCAAC
   TATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTGA

601 CTGGCTCGTTCCTTTATAATTTGCATCCAAATGATTTCAGAAGCAGCAAG
   GACCGAGCAAGGAAATATTAAACGTAGGTTTACTAAAGTCTTCGTCGTTT

651 ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA
   TAAGGTTATATAACTCCCTCTTTACGCGTGCTCTTAATCCATGTTGGCCT

701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGGAGA
```

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**FIGURE 44C (P2)**

CTAGACGTGGTCTAGGATCGCATTAAATGTGAACTCTTATCAACCCCCTCT

751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTTGCTAGTCCAAT  
GAAAGGTGACGTTAAGTTCTCAGATTGGTTCCTCGGAAACGATCAGGTTA

801 TCAACTGCAAAGACGTAATGGTTCCAAATTCAGTGTGTACGATGTGAGTA  
AGTTGACGTTTCTGCATTACCAAGGTTAAGTCACACATGCTACACTCAT

851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGCACCTCCACCA  
ATAATTAGGGATAGTATCGAGAGTACCACATATCTACGCGTGGAGGTGGT

901 TCGTCACAGTTTTCTTTGTCAGCTCTTCTCTCTTCCGATATTTTTAATGC  
AGCAGTGTCAAAGAAACAGTCGAGAAGAGAGAAGGCTATAAAAATTACG

951 TGATGTTGTATGGATCCTGAGCCCATAGTGCGTATCGTAGGTCGAAATG  
ACTACAAACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTTAC

1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA  
CAGATACACAACCTACAATCCCTACCTTCTAAGGTGTTGCCTTTGCGTTAT

1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT  
GTCAACACCGGTACGTTTACGATTATGTCTACGTTTAGTCGAGACCTGAAA

1101 GAAAAGAGACAATACTATTTCGATCTAATGGAAAGTGTTTAACTACTTACG  
CTTTTCTCTGTTATGATAAGCTAGATTACCTTTCACAAATTGATGAATGC

1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA  
CCATGTACAGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT

1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC  
TGACTACGGTGGGCGACCGTTTATACCCTATTACCTTGGTAGTATTTAGG

1251 CAGATCTAGTCTAGTTTTAGCAGCGACATCAGGGAACAGTGGTACCACAC  
GTCTAGATCAGATCAAATCGTCGCTGTAGTCCCTTGTACCATGGTGTG

1301 TTACAGTGCAAACCAACATTTATGCCGTTAGTCAAGGTTGGCTTCCTACT  
AATGTCACGTTTGGTTGTAAATACGGCAATCAGTTCCAACCGAAGGATGA

1351 AATAATACACAACCTTTTTGTTACAACCATTTGTTGGGCTATATGGTCTGTG  
TTATTATGTGTTGGAAAACAATGTTGGTAACAACCCGATATACCAGACAC

1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA  
GAACGTTTCGTTTATCACCTGTTTACATACCTATCTCCTGACATCGTCACTTT

1451 AGGCTGAACAACAGTGGGCTCTTTATGCAGATGGTTCAATACGTCCTCAG  
TCCGACTTGTTGTCACCCGAGAAATACGTCTACCAAGTTATGCAGGAGTC

1501 CAAAACCGAGATAATTGCCTTACAAGTGATTCTAATATACGGGAAACAGT

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**FIGURE 44C (P3)**

GTTTTGGCTCTATTAAACGGAATGTTCACTAAGATTATATGCCCTTTGTCA  
1551 TGTTAAGATCCTCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT  
ACAATTCTAGGAGAGAACACCGGGACGTAGGAGACCGGTTGCTACCTACA  
1601 TCAAGAATGATGGAACCATTTTAAATTTGTATAGTGGATTGGTGTTAGAT  
AGTTCTTACTACCTTGGTAAAATTTAAACATATCACCTAACCACAATCTA  
1651 GTGAGGCGATCGGATCCGAGCCTTAAACAAATCATTCTTTACCCCTCTCCA  
CACTCCGCTAGCCTAGGCTCGGAATTTGTTTAGTAAGAAATGGGAGAGGT  
1701 TGGTGACCCAAACCAAATATGGTTACCATTATTTTGATAGACAGATTACT  
ACCACTGGGTTTGGTTTATACCAATGGTAATAAACTATCTGTCTAATGA  
1751 CTCTTGCAAGTGTGTGTGTCCTGCCATGAAAATAGATGGCTTAAATAAAAA  
GAGAACGTCACACACACAGGACGGTACTTTTATCTACCGAATTTATTTTT  
1801 GGACATTGTAAATTTTGTAAGTAAAGGACAGCAAGTTATATCGAATTCC  
CCTGTAACATTTAAACATTGACTTTCCTGTCGTTCAATATAGCTTAAGG  
1851 TGCAG  
ACGTC

Total number of bases is: 1855.

Sequence name: PAP290

004440 666660



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**FIGURE 44D**

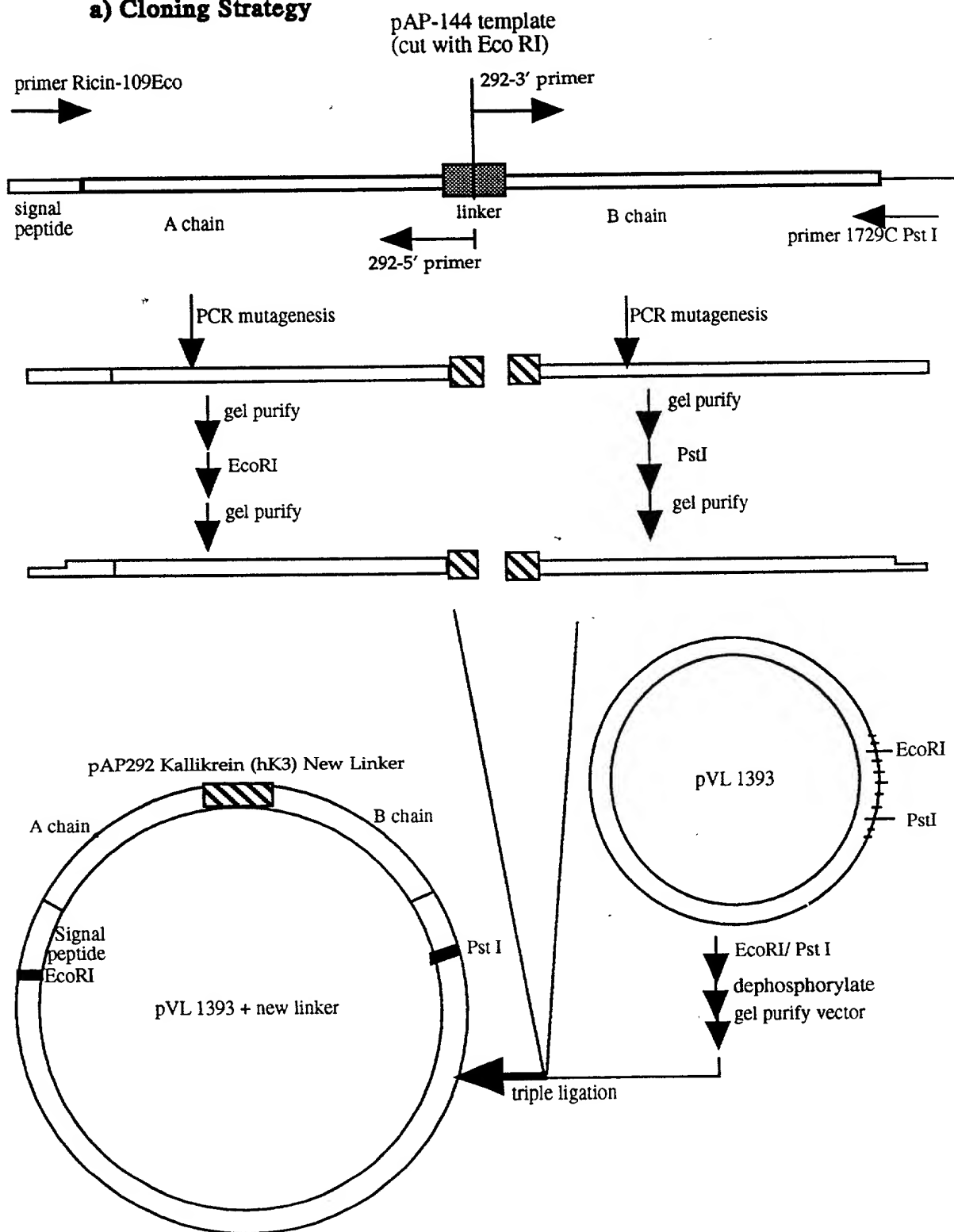
**Amino acid sequence Comparison of Mutant Preproricin Linker  
region of human Prostate-Specific Antigen (PSA) to Wild Type**

Wild type ricin linker:      A chain- S L L I R P V V P N F N -B chain  
pAP-290 (PSA) linker:      A chain- S L S A L L S S D I F N -B chain

004400" 634360

**FIGURE 45A**

**PCR Mutagenesis of Preproricin Gene to Create A Variant Gene in Baculovirus Transfer Vector, pVL 1393**

**a) Cloning Strategy**

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## **FIGURE 45B**

Sequence of Kallikrein (hK3) Linker Region

WT preprocin linker

primer 292-3'  
5' - ATTATCGGTGGCTTTAATGCTGATGTTGT -3'  
\* \*\* \*\*\*\*\*  
-----TCTTTGCTTATAAGGCCA|GTGGTACCAATTTTAAT-----  
-----AGAAACGAATATTCGGT|CACCATGGTTTAAATTA-----  
\* \* \*\*\*\*\*  
3' -AGCAGTGTCAAAGAAACGGATCTAAATTT -5'  
primer 292-5'

1) PCR mutagenesis

2) Ligate with pVL1393

pAP 292 linker  
(Kallikrein variant) -  
-----TCTTTGCCCTAGATTTAAA|ATTATCGGTGGCTTTAAT-----  
-----AGAAACGGATCTAAATTT|TAATAGCCACCGAAATTA-----

004440" F3T360

**FIGURE 45C (P1)**

Sequence of pAP292 insert

	10	20	30	40	50
1	GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT				
	CTTAAGTACTTTGGCCCTCCTTTATGATAACATTATACCTACATACGTCA				
51	GGCAACATGGCTTTGTTTTGGATCCACCTCAGGGTGGTCTTTCACATTAG				
	CCGTTGTACCGAAACAAACCTAGGTGGAGTCCCACCAGAAAGTGTAATC				
101	AGGATAACAACATATTCCCCAAACAATACCCAATTATAAACTTTACCACA				
	TCCTATTGTTGTATAAGGGGTTTGTATGGGTAAATATTGAAATGGTGT				
151	GCGGGTGCCACTGTGCAAAGCTACACAACTTTATCAGAGCTGTTGCGGG				
	CGCCACGGTGACACGTTTCGATGTGTTGAAATAGTCTCGACAAGCGCC				
201	TCGTTTAACAACCTGGAGCTGATGTGAGACATGATATACCAGTGTTGCCAA				
	AGCAAATTGTTGACCTCGACTACACTCTGTACTATATGGTCACAACGGTT				
251	ACAGAGTTGGTTTGCCTATAAACCAACGGTTTATTTAGTTGAACTCTCA				
	TGTCTCAACCAAACGGATATTTGGTTGCCAAATAAAATCAACTTGAGAGT				
301	AATCATGCAGAGCTTTCTGTTACATTAGCGCTGGATGTCACCAATGCATA				
	TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT				
351	TGTGGTCGGCTACCGTGCTGGAAATAGCGCATATTTCTTTCATCCTGACA				
	ACACCAGCCGATGGCAGCAGCTTTATCGCGTATAAAGAAAGTAGGACTGT				
401	ATCAGGAAGATGCAGAAGCAATCACTCATCTTTTCACTGATGTTCAAAT				
	TAGTCCTTCTACGTCTTCGTTAGTGAGTAGAAAAGTGACTACAAGTTTTA				
451	CGATATACATTCGCCCTTTGGTGGTAATTATGATAGACTTGAACAACTTGC				
	GCTATATGTAAGCGGAAACCACCATTAATACTATCTGAACTTGTTGAACG				
501	TGGTAATCTGAGAGAAAATATCGAGTTGGGAAATGGTCCACTAGAGGAGG				
	ACCATTAGACTCTCTTTTATAGCTCAACCCTTTACCAGGTGATCTCCTCC				
551	CTATCTCAGCGCTTTATTATTACAGTACTGGTGGCACTCAGCTTCCAAC				
	GATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA				
601	CTGGCTCGTTTCTTTTATAATTTGCATCCAAATGATTTTCAAGCAGCAAG				
	GACCGAGCAAGGAAATATTAAACGTAGGTTTACTAAAGTCTTCGTCGTT				
651	ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA				
	TAAGGTTATATAACTCCCTCTTTACGCGTGCTCTTAATCCATGTTGGCCT				
701	GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGGAGA				

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FIGURE 45C (P2)

CTAGACGTGGTCTAGGATCGCATTAAATGTGAACTCTTATCAACCCCCTCT  
751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTTGCTAGTCCAAT  
GAAAGGTGACGTTAAGTTCTCAGATTGGTTCCTCGGAAACGATCAGGTTA  
801 TCAACTGCAAAGACGTAATGGTTCCAAATTCAGTGTGTACGATGTGAGTA  
AGTTGACGTTTCTGCATTACCAAGGTTTAAGTCACACATGCTACACTCAT  
851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGCACCTCCACCA  
ATAATTAGGGATAGTATCGAGAGTACCACATATCTACGCGTGGAGGTGGT  
901 TCGTCACAGTTTTCTTTGCCTAGATTTAAAATTATCGGTGGCTTTAATGC  
AGCAGTGTCAAAAGAAACGGATCTAAATTTTAATAGCCACCGAAATTACG  
951 TGATGTTTGTATGGATCCTGAGCCCATAGTGCGTATCGTAGGTCGAAATG  
ACTACAAACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTTAC  
1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA  
CAGATACACAACACTACAATCCCTACCTTCTAAGGTGTTGCCTTTGCGTTAT  
1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT  
GTCAACACCGGTACGTTTCAATTATGTCTACGTTTAGTCGAGACCTGAAA  
1101 GAAAAGAGACAATACTATTTCGATCTAATGGAAAGTGTTTAACTACTTACG  
CTTTTCTCTGTTATGATAAGCTAGATTACCTTTCACAAATTGATGAATGC  
1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA  
CCATGTGACGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT  
1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC  
TGACTACGGTGGGCGACCGTTTATACCCTATTACCTTGGTAGTATTTAGG  
1251 CAGATCTAGTCTAGTTTTAGCAGCGACATCAGGGAACAGTGGTACCACAC  
GTCTAGATCAGATCAAATCGTCGCTGTAGTCCCTTGTCACCATGGTGTG  
1301 TTACAGTGCAAACCAACATTTATGCCGTTAGTCAAGGTTGGCTTCCTACT  
AATGTCACGTTTGGTTGTAAATACGGCAATCAGTTCCAACCGAAGGATGA  
1351 AATAATACACAACCTTTTGTTACAACCATTTGTTGGGCTATATGGTCTGTG  
TTATTATGTGTTGGAAAACAATGTTGGTAACAACCCGATATACCAGACAC  
1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA  
GAACGTTTCGTTTATCACCTGTTTATACCTATCTCCTGACATCGTCACTTT  
1451 AGGCTGAACAACAGTGGGCTCTTTATGCAGATGGTTCAATACGTCCTCAG  
TCCGACTTGTGTCACCCGAGAAATACGTCTACCAAGTTATGCAGGAGTC  
1501 CAAAACCGAGATAATTGCCTTACAAGTGATTCTAATATACGGGAAACAGT

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**FIGURE 45C (P3)**

GTTTTGGCTCTATTAACGGAATGTTCACTAAGATTATATGCCCTTTGTCA  
1551 TGTTAAGATCCTCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT  
ACAATTCTAGGAGAGAACACCGGGACGTAGGAGACCGGTTGCTACCTACA  
1601 TCAAGAATGATGGAACCATTTTAAATTTGTATAGTGGATTGGTGTTAGAT  
AGTTCTTACTACCTTGGTAAAATTTAAACATATCACCTAACCACAATCTA  
1651 GTGAGGCGATCGGATCCGAGCCTTAAACAAATCATTCTTTACCCTCTCCA  
CACTCCGCTAGCCTAGGCTCGGAATTTGTTTAGTAAGAAATGGGAGAGGT  
1701 TGGTGACCCAAACCAATATGGTTACCATTATTTTGATAGACAGATTACT  
ACCACTGGGTTTGGTTTATACCAATGGTAATAAACTATCTGTCTAATGA  
1751 CTCTTGCA GTGTGTGTGTCCTGCCATGAAAATAGATGGCTTAAATAAAAA  
GAGAACGTCACACACACAGGACGGTACTTTTATCTACCGAATTTATTTTT  
1801 GGACATTGTAAATTTTGTAACTGAAAGGACAGCAAGTTATATCGAATTCC  
CCTGTAACATTTAAACATTGACTTTCCTGTCGTTCAATATAGCTTAAGG  
1851 TGCAG  
ACGTC

Total number of bases is: 1855.

Sequence name: PAP292

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**FIGURE 45D**

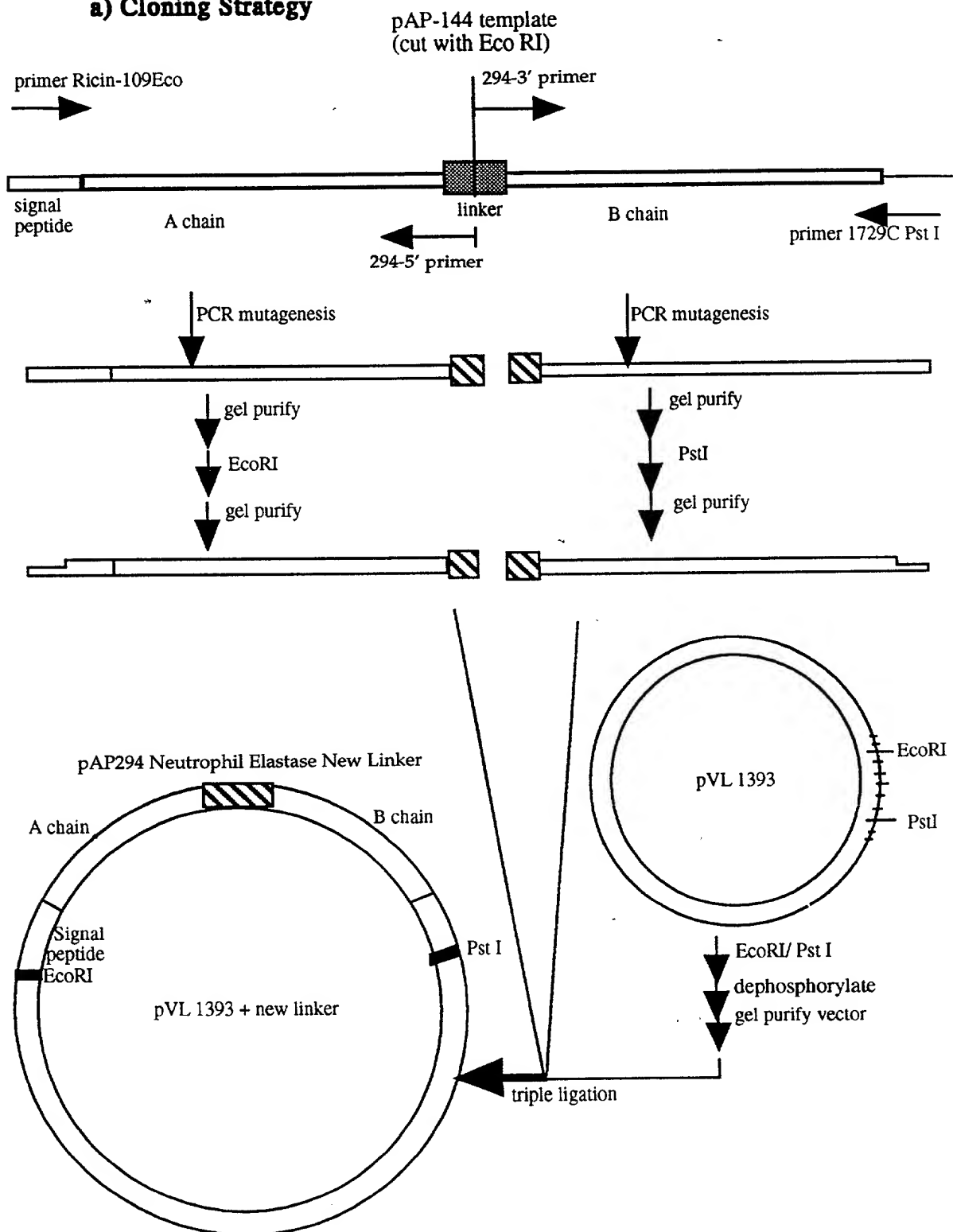
Amino acid sequence Comparison of Mutant Preproricin Linker  
region of Kallikrein (hK3) to Wild Type

Wild type ricin linker:      A chain- S L L I R P V V P N F N -B chain  
pAP-292 (hK3) linker:      A chain- S L P R F K I I G G F N -B chain

00440" 157660

**FIGURE 46A**

**PCR Mutagenesis of Preproricin Gene to Create A Variant Gene in Baculovirus Transfer Vector, pVL 1393**

**a) Cloning Strategy**



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## FIGURE 46B

Sequence of Neutrophil Elastase Linker Region

### **WT preprocin linker**

primer 294-3'

5'- GTTCCTGGTAATTTTAATGCTGATGTTTGT -3'

\*\* \*\*\*\*\*

-----TCTTTGCTTATAAGGCCA|GTGGTACCAAATTTTAAT-----  
-----AGAAACGAATATTCCGGT|CACCATGGTTTAAAATTA-----  
\*\*\* \*\*\* \*

3'-AGCAGTGTCAAAAGAAACGAACCGTAACGA -5'

primer 294-5'

1) PCR mutagenesis

2) Ligate with pVL1393

pAP 294 linker

(Neutrophil elastase variant)

-----TCTTTGCTTGGCATTGCT|GTTCCTGGTAATTTTAAT-----  
-----AGAAACGAACCGTAACGA|CAAGGACCATTAAAATTA-----

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# FIGURE 46C (P1)

Sequence of pAP294 insert

10 20 30 40 50  
| | | | |  
1 GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT  
CTTAAGTACTTTGGCCCTCCTTTATGATAACATTATACCTACATACGTCA  
51 GGCAACATGGCTTTGTTTTGGATCCACCTCAGGGTGGTCTTTCACATTAG  
CCGTTGTACCGAAACAAACCTAGGTGGAGTCCCACCAGAAAGTGTAATC  
101 AGGATAACAACATATTCCCCAAACAATACCCAATTATAAACTTTACCACA  
TCCTATTGTTGTATAAGGGGTTTGTATGGGTAAATATTGAAATGGTGT  
151 GCGGGTGCCACTGTGCAAAGCTACACAACTTTATCAGAGCTGTTGCGGG  
CGCCACGGTGACACGTTTCGATGTGTTTGAAATAGTCTCGACAAGCGCC  
201 TCGTTTAACTGAGCTGATGTGAGACATGATATACCAGTGTGCCAA  
AGCAAATTGTTGACCTCGACTACACTCTGTACTATATGGTCACAACGGTT  
251 ACAGAGTTGGTTTGCCTATAAACCAACGGTTTATTTAGTTGAACTCTCA  
TGTCTCAACCAACGGATATTGGTTGCCAAATAAAATCAACTTGAGAGT  
301 AATCATGCAGAGCTTCTGTTACATTAGCGCTGGATGTCACCAATGCATA  
TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT  
351 TGTGGTCGGCTACCGTGCTGGAAATAGCGCATATTTCTTTCATCCTGACA  
ACACCAGCCGATGGCAGACCTTTATCGCGTATAAAGAAAGTAGGACTGT  
401 ATCAGGAAGATGCAGAAGCAATCACTCATCTTTTCACTGATGTTCAAAT  
TAGTCCTTCTACGTCTTCGTTAGTGAGTAGAAAAGTGACTACAAGTTTTA  
451 CGATATACATTCGCCTTTGGTGGTAATTATGATAGACTTGAACAACCTGC  
GCTATATGTAAGCGGAAACCACCATTAACTATCTGAACCTGTTGAACG  
501 TGGTAATCTGAGAGAAAATATCGAGTTGGGAAATGGTCCACTAGAGGAGG  
ACCATTAGACTCTCTTTTATAGCTCAACCCTTTACCAGGTGATCTCCTCC  
551 CTATCTCAGCGCTTTATTATTACAGTACTGGTGGCACTCAGCTTCCAAC  
GATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA  
601 CTGGCTCGTTCCTTTATAATTTGCATCCAAATGATTTCAGAAGCAGCAAG  
GACCGAGCAAGGAAATATTAAACGTAGGTTTACTAAAGTCTTCGTCGTT  
651 ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA  
TAAGGTTATATAACTCCCTCTTACGCGTGCTCTTAATCCATGTTGGCCT  
701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGGAGA

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**FIGURE 46C (P2)**

CTAGACGTGGTCTAGGATCGCATTAAATGTGAACTCTTATCAACCCCCCTCT  
751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTTGCTAGTCCAAT  
GAAAGGTGACGTTAAGTTCTCAGATTGGTTCCTCGGAAACGATCAGGTTA  
801 TCAACTGCAAAGACGTAATGGTTCCAAATTCAGTGTGTACGATGTGAGTA  
AGTTGACGTTTCTGCATTACCAAGGTTTAAGTCACACATGCTACACTCAT  
851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGCACCTCCACCA  
ATAATTAGGGATAGTATCGAGAGTACCACATATCTACGCGTGGAGGTGGT  
901 TCGTCACAGTTTTCTTTGCTTGGCATTGCTGTTCCCTGGTAATTTTAATGC  
AGCAGTGTCAAAGAAACGAACCGTAACGACAAGGACCATTAAAATTACG  
951 TGATGTTTGTATGGATCCTGAGCCCATAGTGCGTATCGTAGGTCGAAATG  
ACTACAAACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTTAC  
1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA  
CAGATACACAACCTACAATCCCTACCTTCTAAGGTGTTGCCTTTGCGTTAT  
1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT  
GTCAACACCGGTACGTTTCAAGTTATGTCTACGTTTAGTCGAGACCTGAAA  
1101 GAAAAGAGACAATACTATTGATCTAATGGAAAGTGTTTAACTACTTACG  
CTTTTCTCTGTTATGATAAGCTAGATTACCTTTCACAAATTGATGAATGC  
1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA  
CCATGTCAGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT  
1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC  
TGACTACGGTGGGCGACCGTTTATACCCTATTACCTTGGTAGTATTTAGG  
1251 CAGATCTAGTCTAGTTTTAGCAGCGACATCAGGGAACAGTGGTACCACAC  
GTCTAGATCAGATCAAATCGTCGCTGTAGTCCCTTGTCACCATGGTGTG  
1301 TTACAGTGCAAACCAACATTTATGCCGTTAGTCAAGGTGGCTTCCTACT  
AATGTCACGTTTGGTTGTAAATACGGCAATCAGTTCCAACCGAAGGATGA  
1351 AATAATACACAACCTTTTGTTACAACCATTTGTTGGGCTATATGGTCTGTG  
TTATTATGTGTTGGAAAACAATGTTGGTAACAACCCGATATACCAGACAC  
1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA  
GAACGTTTCGTTTATCACCTGTTTCATACCTATCTCCTGACATCGTCACTTT  
1451 AGGCTGAACAACAGTGGGCTCTTTATGCAGATGGTTCAATACGTCCTCAG  
TCCGACTTGTGTACCCGAGAAATACGTCTACCAAGTTATGCAGGAGTC  
1501 CAAAACCGAGATAATTGCCTTACAAGTGATTCTAATATACGGGAAACAGT

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**FIGURE 46C (P3)**

GTTTTGGCTCTATTAAACGGAATGTTCACTAAGATTATATGCCCTTTGTCA  
 1551 TGTTAAGATCCTCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT  
 ACAATTCTAGGAGAGAACACCGGGACGTAGGAGACCGGTTGCTACCTACA  
 1601 TCAAGAATGATGGAACCATTTTAAATTTGTATAGTGGATTGGTGTTAGAT  
 AGTTCTTACTACCTTGGTAAAATTTAAACATATCACCTAACCACAATCTA  
 1651 GTGAGGCGATCGGATCCGAGCCTTAAACAAATCATTCTTTACCCCTCTCCA  
 CACTCCGCTAGCCTAGGCTCGGAATTTGTTTAGTAAGAAATGGGAGAGGT  
 1701 TGGTGACCCAAACCAAATATGGTTACCATTATTTTGATAGACAGATTACT  
 ACCACTGGGTTTGGTTTATACCAATGGTAATAAAACTATCTGTCTAATGA  
 1751 CTCTTGCAGTGTGTGTGTCCTGCCATGAAAATAGATGGCTTAAATAAAAA  
 GAGAACGTACACACACAGGACGGTACTTTTATCTACCGAATTTATTTTT  
 1801 GGACATTGTAAATTTTGTAAGTGAAGGACAGCAAGTTATATCGAATTCC  
 CCTGTAACATTTAAACATTGACTTTCCTGTCGTTCAATATAGCTTAAGG  
 1851 TGCAG  
 ACGTC

Total number of bases is: 1855.

Sequence name: PAP294

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**FIGURE 46D**

**Amino acid sequence Comparison of Mutant Preproricin Linker  
region of Neutrophil elastase to Wild Type**

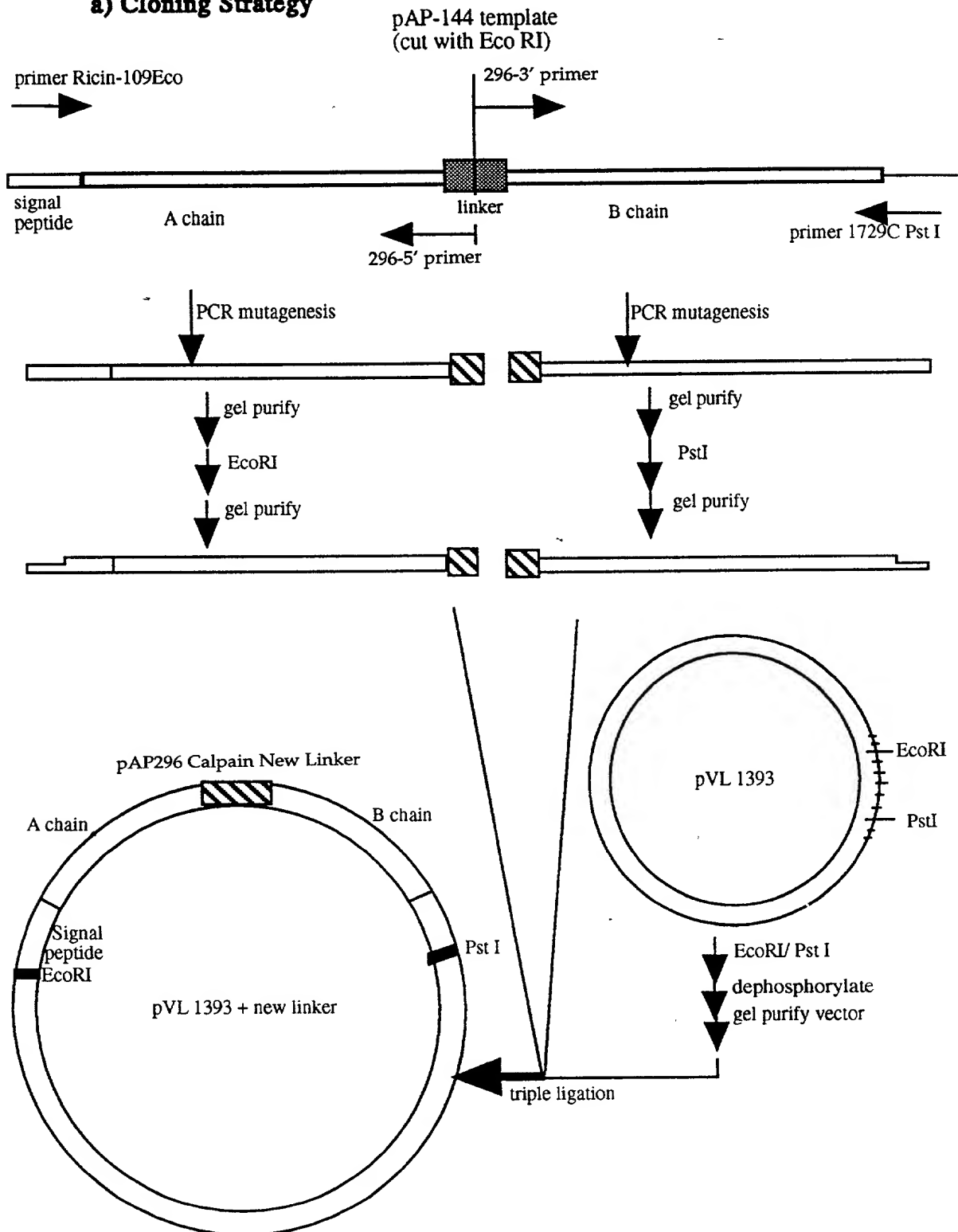
**Wild type ricin linker:           A chain- S L L I R P V V P N F N -B chain**

**pAP-294 (Neutrophil elastase) linker:  
                                  A chain- S L L G I A V P G N F N -B chain**

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**FIGURE 47A**

**PCR Mutagenesis of Preproricin Gene to Create A Variant Gene in Baculovirus Transfer Vector, pVL 1393**

**a) Cloning Strategy**

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## FIGURE 47B

### Sequence of Calpain Linker Region

#### WT preprocin linker

primer 296-3'  
5'- ACTCCTAGAACCCCCCAGCTGATGTTTGT -3'  
\*\*\*\*\*  
-----TCCTTGCTTATAAGGCCA|GTGGTACCAAATTTTAAT-----  
-----AGAAACGAATATTCCGGT|CACCATGGTTTAAATTA-----  
\*     \* \* \* \* \*  
3'-AGCAGTGTCAAAAAAAGTTTTATAACAA -5'  
primer 296-5'

1) PCR mutagenesis

2) Ligate with pVL1393

pAP 296 linker  
(Calpain variant)

-----TTTTTCAAAAATATTGTT|ACTCCTAGAACCCCCCA-----  
-----AAAAAGTTTTTATAACAA|TGAGGATCTTGGGGGGGT-----

**FIGURE 47C (P1)**

Sequence of pAP296 insert

```

      10      20      30      40      50
      |      |      |      |      |
1  GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT
   CTTAAGTACTTTGGCCCTCCTTTATGATAACATTATACCTACATACGTCA

51  GGCAACATGGCTTTGTTTTGGATCCACCTCAGGGTGGTCTTTCACATTAG
   CCGTTGTACCGAAACAAAACCTAGGTGGAGTCCCACCAGAAAGTGTAATC

101 AGGATAACAACATATTCCCCAAACAATACCCAATTATAAACTTTACCACA
   TCCTATTGTTGTATAAGGGGTTTGTATGGGTAAATATTTGAAATGGTGT

151 GCGGGTGCCACTGTGCAAAGCTACACAACTTTATCAGAGCTGTTGCGGG
   CGCCACGCGTGACACGTTTCGATGTGTTTGAAATAGTCTCGACAAGCGCC

201 TCGTTTAACAACCTGGAGCTGATGTGAGACATGATATAACAGTGTTGCCAA
   AGCAAATTGTTGACCTCGACTACACTCTGTACTATATGGTCACAACGGTT

251 ACAGAGTTGGTTTGCCTATAAACCAACGGTTTATTTTAGTTGAACTCTCA
   TGTCTCAACCAAACGGATATTTGGTTGCCAAATAAAATCAACTTGAGAGT

301 AATCATGCAGAGCTTTCTGTTACATTAGCGCTGGATGTCACCAATGCATA
   TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT

351 TGTGGTCGGCTACCGTGCTGGAAATAGCGCATATTTCTTTCATCCTGACA
   ACACCAGCCGATGGCACGACCTTTATCGCGTATAAAGAAAGTAGGACTGT

401 ATCAGGAAGATGCAGAAGCAATCACTCATCTTTTCACTGATGTTCAAAT
   TAGTCCTTCTACGTCTTCGTTAGTGAGTAGAAAAGTGACTACAAGTTTTA

451 CGATATACATTTCGCCTTTGGTGGTAATTATGATAGACTTGAACAACTTGC
   GCTATATGTAAGCGGAAACCACCATTAACTATCTGAACCTGTTGAACG

501 TGGTAATCTGAGAGAAAATATCGAGTTGGGAAATGGTCCACTAGAGGAGG
   ACCATTAGACTCTCTTTTATAGCTCAACCCTTTACCAGGTGATCTCCTCC

551 CTATCTCAGCGCTTTATTATTACAGTACTGGTGGCACTCAGCTTCCAAC
   TATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA

601 CTGGCTCGTTCCTTTATAATTTGCATCCAAATGATTTCAGAAGCAGCAAG
   GACCGAGCAAGGAAATATTAAACGTAGGTTTACTAAAGTCTTCGTCGTTG

651 ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA
   TAAGGTTATATAACTCCCTCTTTACGCGTGCTCTTAATCCATGTTGGCCT

701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGGAGA
```



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FIGURE 47C (P2)

CTAGACGTGGTCTAGGATCGCATTAAATGTGAACTCTTATCAACCCCCCTCT

751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTTGCTAGTCCAAT  
GAAAGGTGACGTTAAGTTCTCAGATTGGTTCCTCGGAAACGATCAGGTTA

801 TCAACTGCAAAGACGTAATGGTTCCAAATTCAGTGTGTACGATGTGAGTA  
AGTTGACGTTTCTGCATTACCAAGGTTTAAGTCACACATGCTACACTCAT

851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGCACCTCCACCA  
ATAATTAGGGATAGTATCGAGAGTACCACATATCTACGCGTGGAGGTGGT

901 TCGTCACAGTTTTTTTTTCAAAAATATTGTTACTCCTAGAACCCCCCAGC  
AGCAGTGTCAAAAAAAGTTTTTATAACAATGAGGATCTTGGGGGGGTGCG

951 TGATGTTTGTATGGATCCTGAGCCCATAGTGCCTATCGTAGGTCGAAATG  
ACTACAAACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTTAC

1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA  
CAGATACACAAC TACAATCCCTACCTTCTAAGGTGTTGCCTTTGCGTTAT

1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT  
GTCAACACCGGTACGTTTCAAGATTATGTCTACGTTTAGTCGAGACCTGAAA

1101 GAAAAGAGACAATACTATTCGATCTAATGGAAAGTGTTTAACTACTTACG  
CTTTTCTCTGTTATGATAAGCTAGATTACCTTTCACAAATTGATGAATGC

1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA  
CCATGTCAGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT

1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC  
TGACTACGGTGGGCGACCGTTTATACCCTATTACCTTGGTAGTATTTAGG

1251 CAGATCTAGTCTAGTTTTAGCAGCGACATCAGGGAACAGTGGTACCACAC  
GTCTAGATCAGATCAAAATCGTCGCTGTAGTCCCTGTCACCATGGTGTG

1301 TTACAGTGCAAACCAACATTTATGCCGTTAGTCAAGGTTGGCTTCCTACT  
AATGTCACGTTTGGTTGTAAATACGGCAATCAGTTCCAACCGAAGGATGA

1351 AATAATACACAACCTTTTTGTTACAACCATTGTTGGGCTATATGGTCTGTG  
TTATTATGTGTTGGAAAACAATGTTGGTAACAACCCGATATACCAGACAC

1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA  
GAACGTTTCGTTTATCACCTGTTTCATACCTATCTCCTGACATCGTCACTTT

1451 AGGCTGAACAACAGTGGGCTCTTTATGCAGATGGTTCAATACGTCCTCAG  
TCCGACTTGTTGTCACCCGAGAAATACGTCTACCAAGTTATGCAGGAGTC

1501 CAAAACCGAGATAATTGCCTTACAAGTGATTCTAATATACGGGAAACAGT

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FIGURE 47C (P3)

GTTTTGGCTCTATTAACGGAATGTTCACTAAGATTATATGCCCTTTGTCA  
1551 TGTTAAGATCCTCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT  
ACAATTCTAGGAGAGAACACCGGGACGTAGGAGACCGGTTGCTACCTACA  
1601 TCAAGAATGATGGAACCATTTTAAATTTGTATAGTGGATTGGTGTTAGAT  
AGTTCTTACTACCTTGGTAAAATTTAAACATATCACCTAACCACAATCTA  
1651 GTGAGGCGATCGGATCCGAGCCTTAAACAAATCATTCTTTACCCTCTCCA  
CACTCCGCTAGCCTAGGCTCGGAATTTGTTTAGTAAGAAATGGGAGAGGT  
1701 TGGTGACCCAAACCAAATATGGTTACCATTATTTTGATAGACAGATTACT  
ACCACTGGGTTTGGTTTATACCAATGGTAATAAACTATCTGTCTAATGA  
1751 CTCTTGCAGTGTGTGTGTCCTGCCATGAAAATAGATGGCTTAAATAAAAA  
GAGAACGTCACACACACAGGACGGTACTTTTATCTACCGAATTTATTTTT  
1801 GGACATTGTAAATTTTGTAAGTAAAGGACAGCAAGTTATATCGAATTCC  
CCTGTAACATTTAAACATTGACTTTCCTGTCGTTCAATATAGCTTAAGG  
1851 TGCAG  
ACGTC

Total number of bases is: 1855.

Sequence name: PAP296

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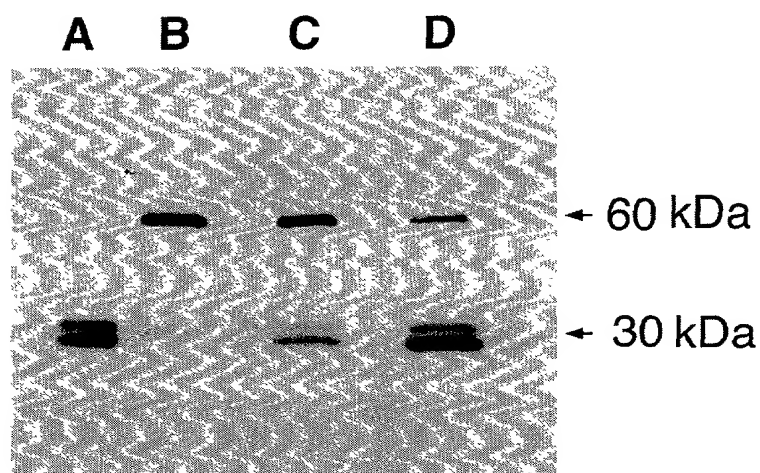
**FIGURE 47D**

**Amino acid sequence Comparison of Mutant Preproricin Linker  
region of Calpain to Wild Type**

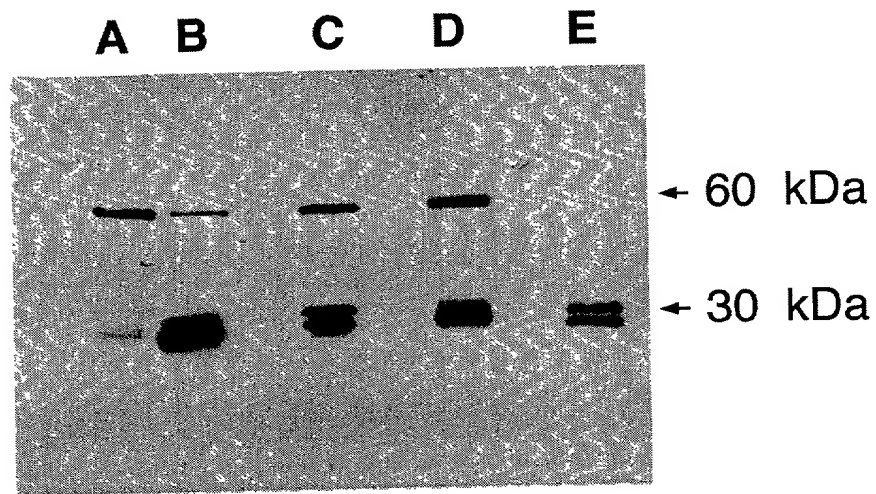
**Wild type ricin linker:           A chain- S L L I R P V V P N F N -B chain**

**pAP-296 (Calpain) linker:       A chain- F F K N I V T P R T P P -B chain**

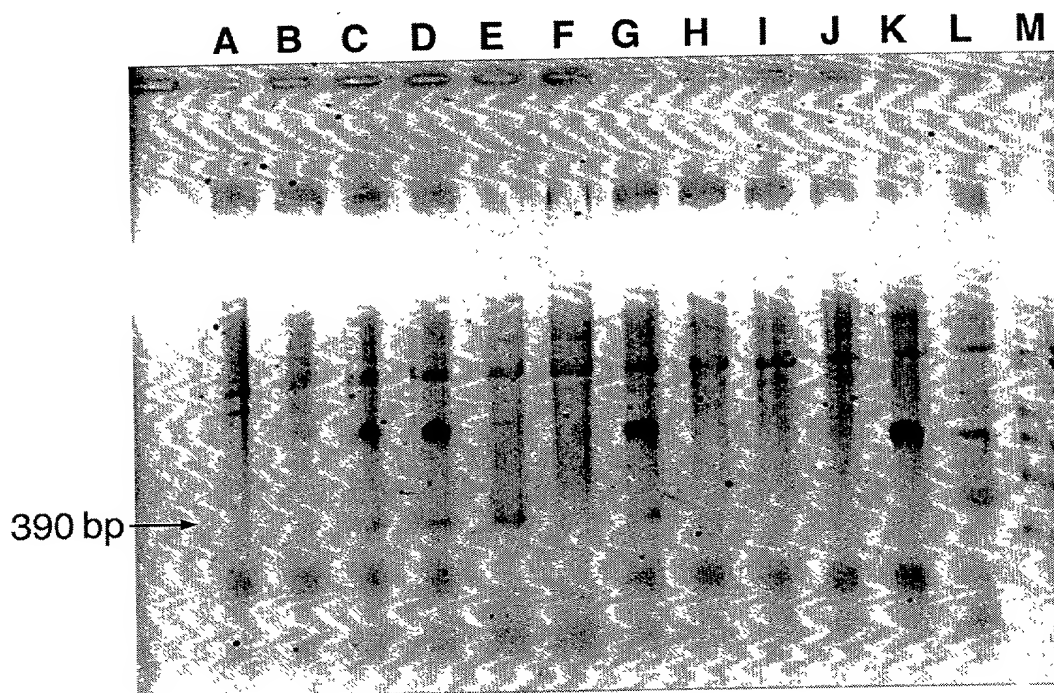
00440" FEF360

**FIGURE 48****Cleavage of pAP 214 by Cathepsin B**

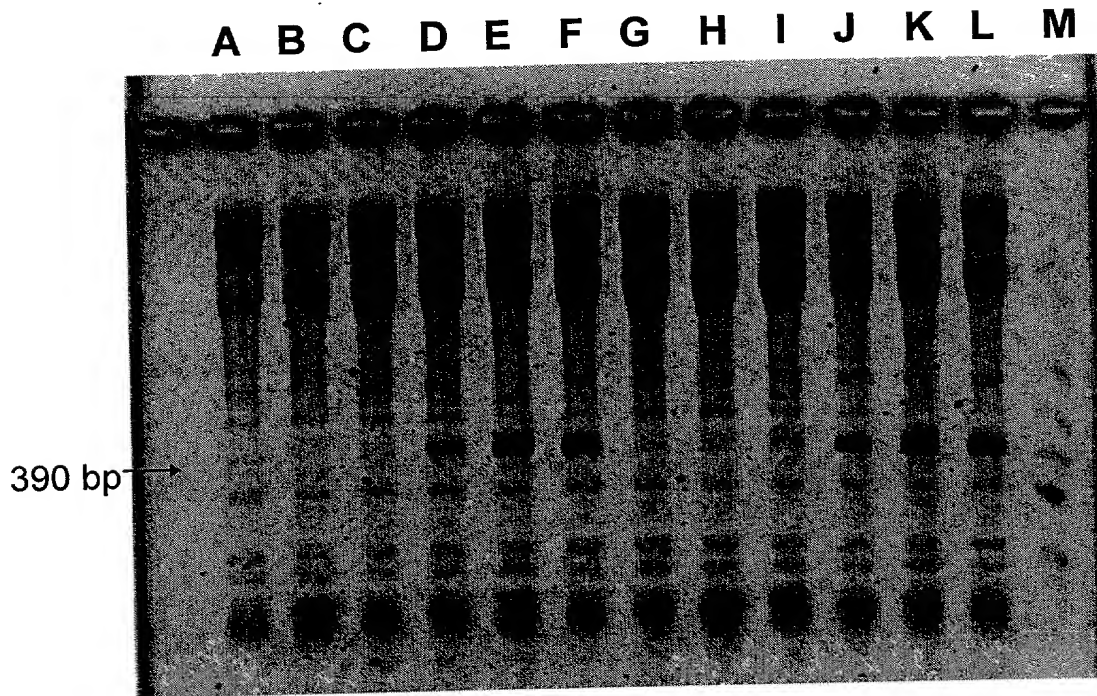
- A.** Ricin standard
- B.** pAP 214
- C.** pAP 214 digested with 100 ng of Cathepsin B (18 hours)
- D.** pAP 214 digested with 618 ng of Cathepsin B (18 hours)

**FIGURE 49****Cleavage of pAP 220 with MMP-9**

- A.** pAP 220
- B.** pAP 220 digested with 200 ng of MMP-9 (16 hrs)
- C.** pAP 220 digested with 20 ng of MMP-9 (16hrs)
- D.** pAP 220 digested with 20 ng of MMP-9 (2hrs)

**FIGURE 50****Activation of pAP 214**

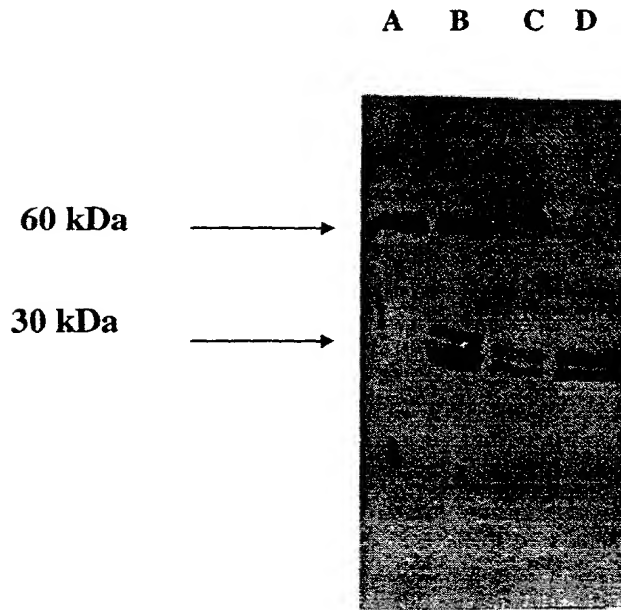
- A.** 41.7 pg of pAP 214 digested with Cathepsin B
- B.** 291 pg of pAP 214 digested with Cathepsin B
- C.** 2.0 ng of pAP 214 digested with Cathepsin B
- D.** 14.2 ng of pAP 214 digested with Cathepsin B
- E.** 100 ng of pAP 214 digested with Cathepsin B
- F.** Negative control
- G.** Ricin A chain
- H.** 41.7 pg of pAP 214 variant
- I.** 291 pg of pAP 214 variant
- J.** 2.0 ng of pAP 214 variant
- K.** 14.2 ng of pAP 214 variant
- L.** 100ng of pAP 214 variant
- M.** RNA ladder

**FIGURE 51****Activation of pAP 220**

- A.** 48.5 pg of pAP 220 variant
- B.** 291 pg of pAP 220 variant
- C.** 2.0 ng of pAP 220 variant
- D.** 14.3 ng of pAP 220 variant
- E.** 100 ng of pAP 220 variant
- F.** Ricin A chain
- G.** Negative Control
- H.** 48.5 pg of pAP 220 variant digested with MMP-9
- I.** 291 pg of pAP 220 variant digested with MMP-9
- J.** 2.0 ng of pAP 220 variant digested with MMP-9
- K.** 14.3 ng of pAP 220 variant digested with MMP-9
- L.** 100 ng of pAP 220 variant digested with MMP-9
- M.** RNA ladder

# FIGURE 52

**Cleavage of pAP-248 Protein by The Human Cytomegalovirus (HCMV) protease**



- A. pAP-248 (0.279 ug)
- B. pAP-248 protein (0.279  $\mu$ g) digested with 0.25  $\mu$ g of the HCMV protease
- C. Ricin standard (20 ng)
- D. Ricin standard (40 ng)

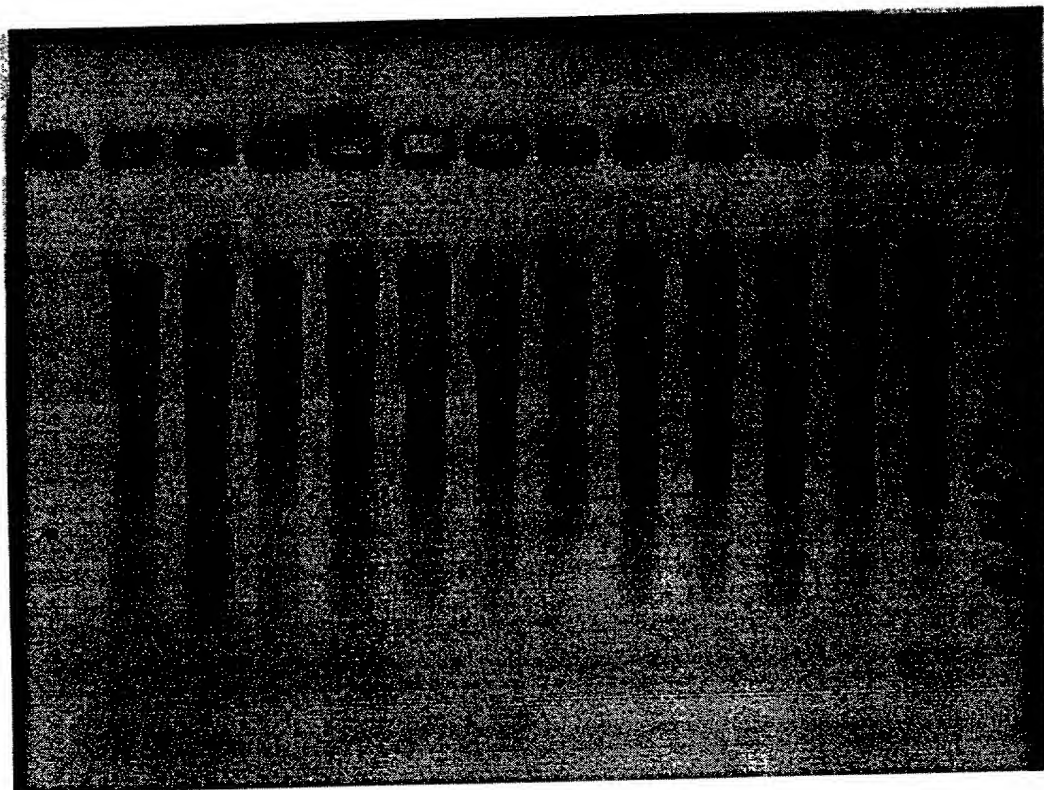


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## FIGURE 53

### Activation of pAP-248 Protein

A B C D E F G H I J K L M

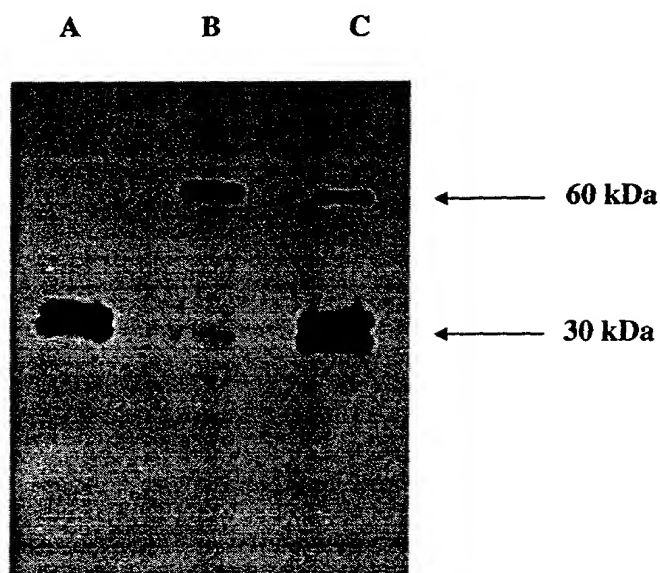


390 b fragment

- A. 90 ng of pAP-248 variant
- B. 12.8 ng of pAP-248 variant
- C. 1.8 ng of pAP-248 variant
- D. 260 pg pAP-248 variant
- E. 37 pg of pAP-248 variant
- F. Negative control
- G. Ricin A chain
- H. 37 pg of pAP-248 digested with HCMV protease
- I. 260 pg of pAP-248 digested with HCMV protease
- J. 1.8 ng of pAP-248 digested with HCMV protease
- K. 12.8 ng of pAP-248 digested with HCMV protease
- L. 90 ng of pAP-248 digested with HCMV protease
- M. RNA ladder

# FIGURE 54

**Cleavage of pAP-256 protein by The Hepatitis A Virus 3C (HAV 3C) Protease**

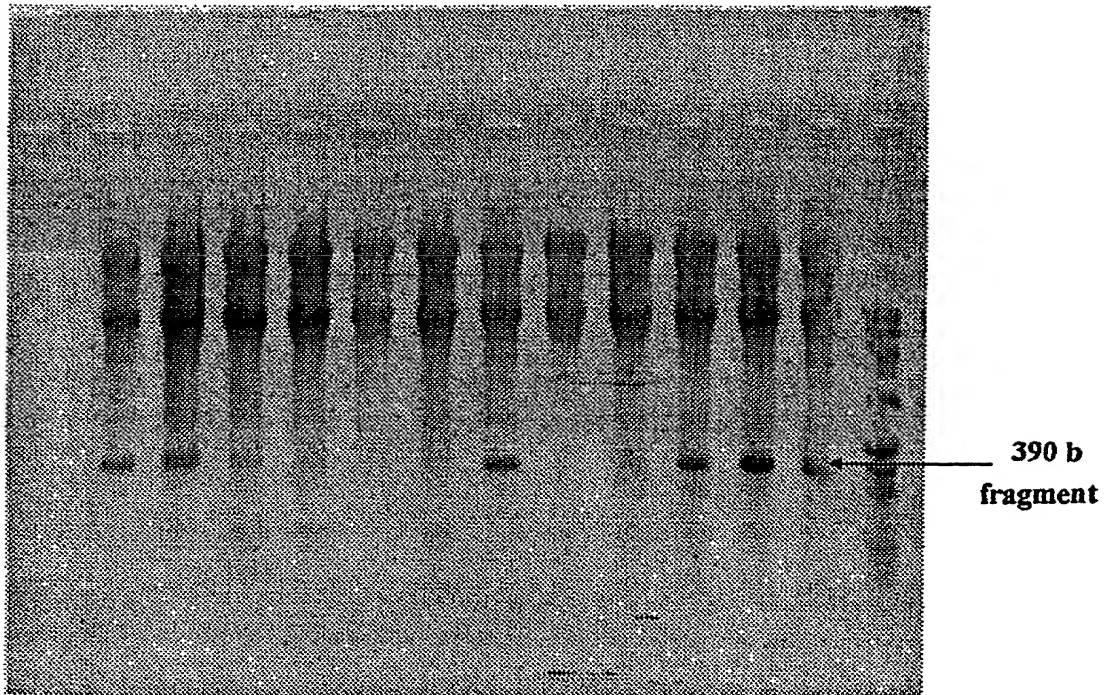


- A. Ricin standard (0.250 ug)**
- B. pAP-256 protein (0.378 ug)**
- C. pAP-256 protein digested (0.302 ug) with 1.25 μg of the HAV 3C protease**

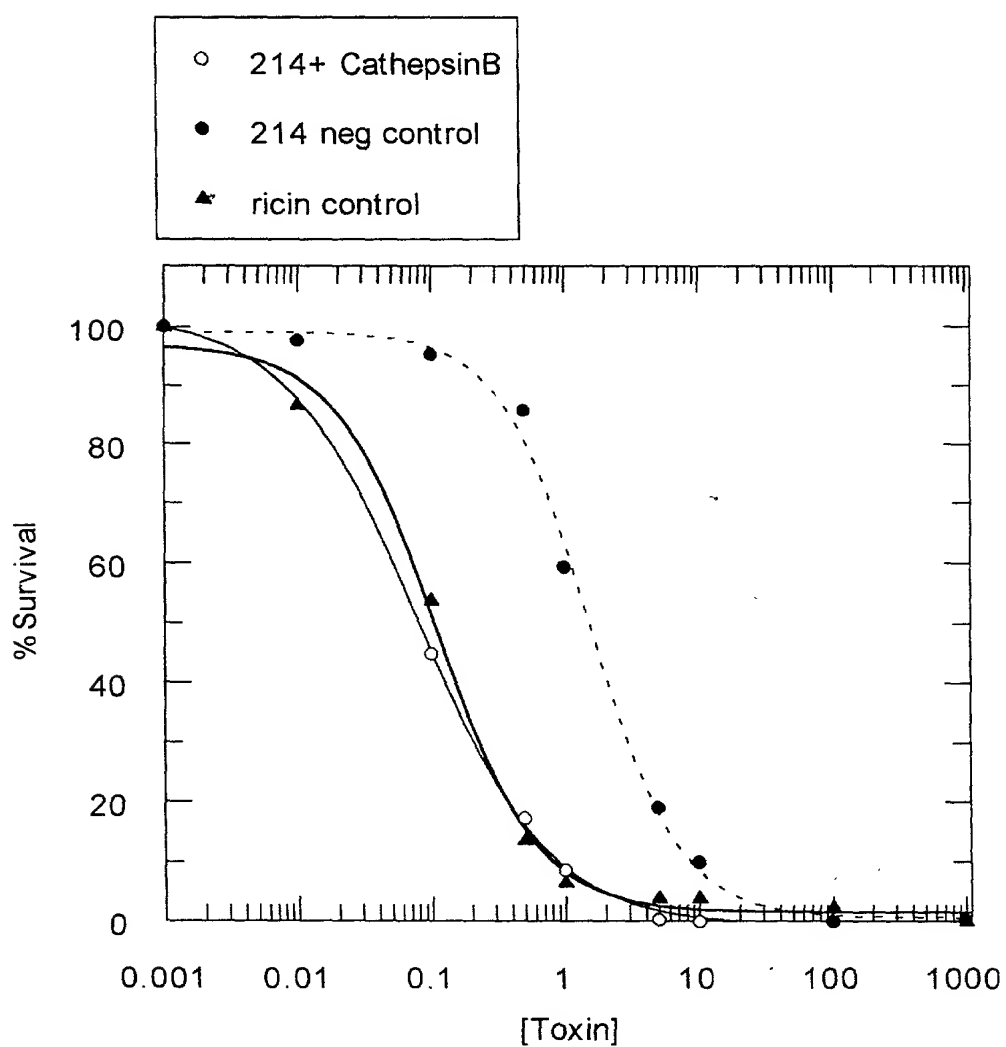
# FIGURE 55

## Activation of pAP-256 Protein

A B C D E F G H I J K L M



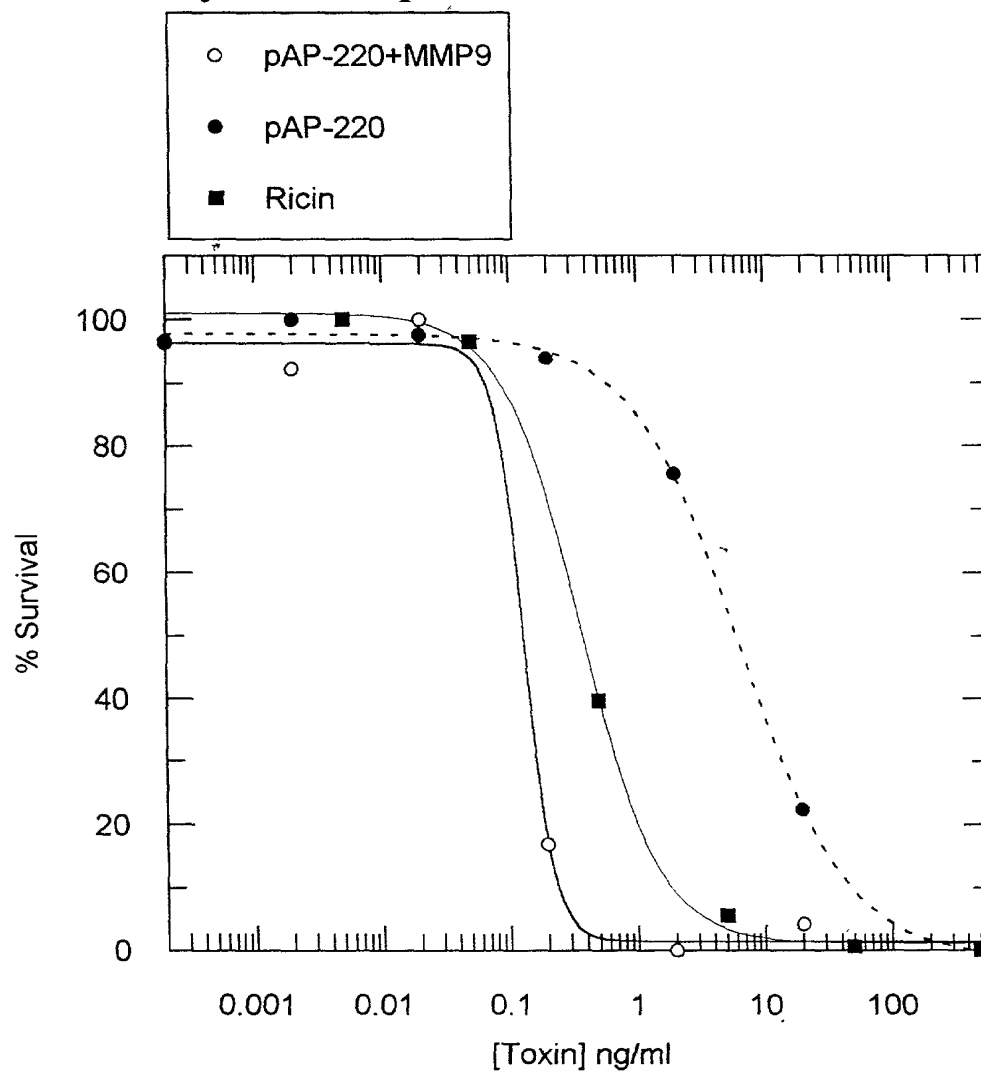
- A. 100 ng of pAP-256 variant
- B. 14.2 ng of pAP-256 variant
- C. 2.0 ng of pAP-256 variant
- D. 291 pg of pAP-256 variant
- E. 41.7 pg of pAP-256 variant
- F. Negative control
- G. Ricin A chain
- H. 41.7 pg of pAP-256 digested with HAV 3C protease
- I. 291 pg of pAP-256 digested with HAV 3C protease
- J. 2.0 ng of pAP-256 digested with HAV 3C protease
- K. 14.2 ng of pAP-256 digested with HAV 3C protease
- L. 100 ng of pAP-256 digested with HAV 3C protease
- M. RNA ladder

**FIGURE 56****Cytotoxicity of Digested and Undigested  
pAP 214 with Cathepsin B to COS-1 Cells**

	Ricin	pAP 214	pAP 214 + Cathepsin B
IC <sub>50</sub> (ng/ml)	0.11	1.9	0.078
Relative Toxicity	1X	17X	0.7X

**FIGURE 57**

**Cytotoxicity of pAP220 Digested with MMP-9 Compared to Freshly Thawed pAP220 and Ricin on COS-1 Cells**

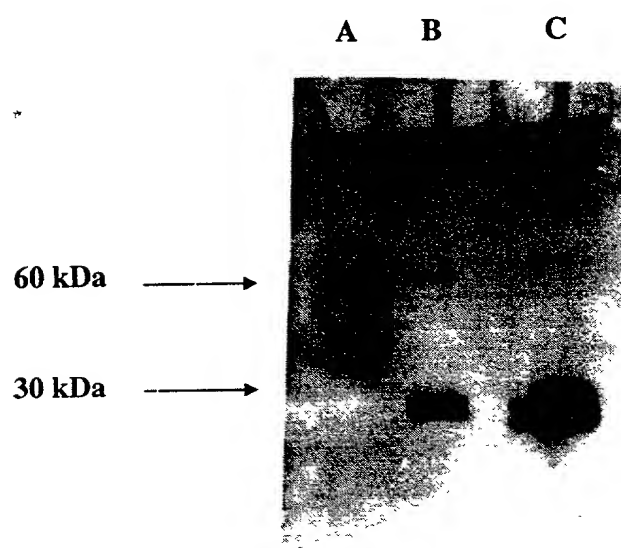


	Ricin	pAP 220	pAP 220 + MMP-9
IC <sub>50</sub> (ng/ml)	0.31	6.7	0.13
Relative Toxicity	1X	22X	0.4X

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## **FIGURE 58**

**Cleavage of pAP-270 protein by The Matrix Metalloproteinase 2 (MMP-2)**



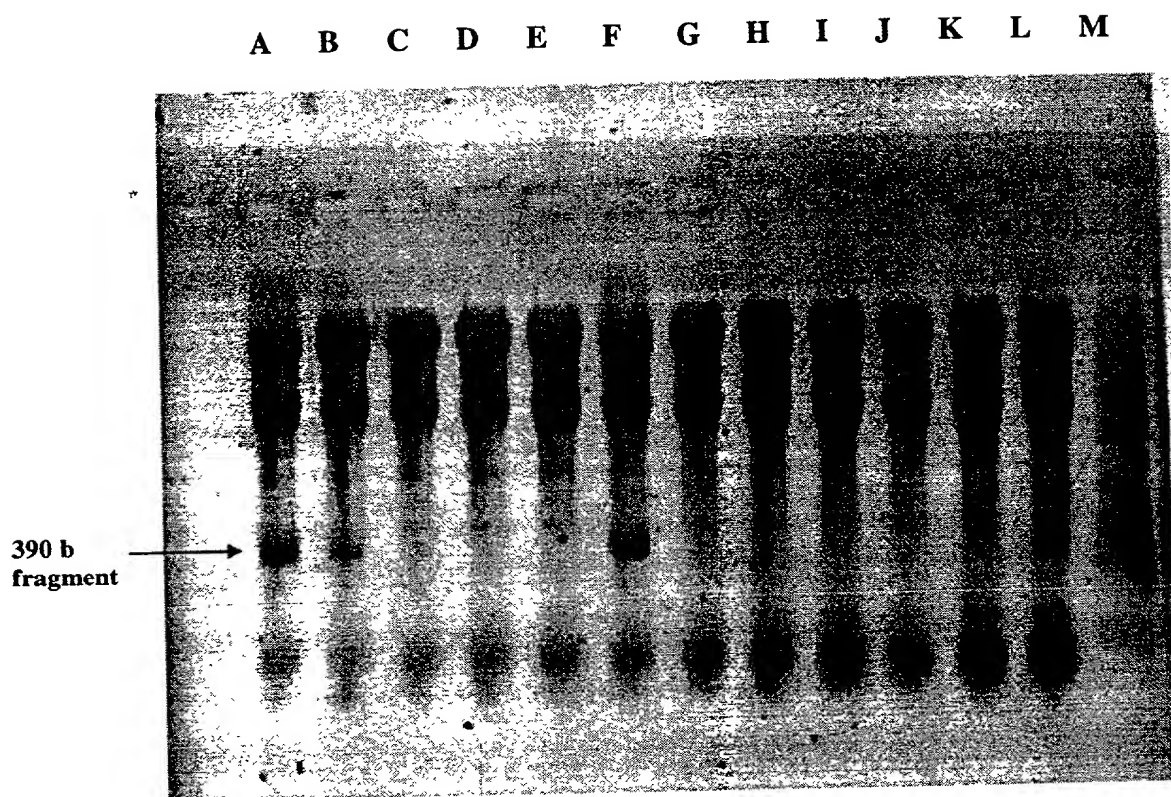
**A. pAP-270 (0.120 µg) undigested**

**B. pAP-270 (0.120 µg) digested with 0.250 µg MMP-2**

**C. Ricin Standard (0.05 µg)**

# FIGURE 59

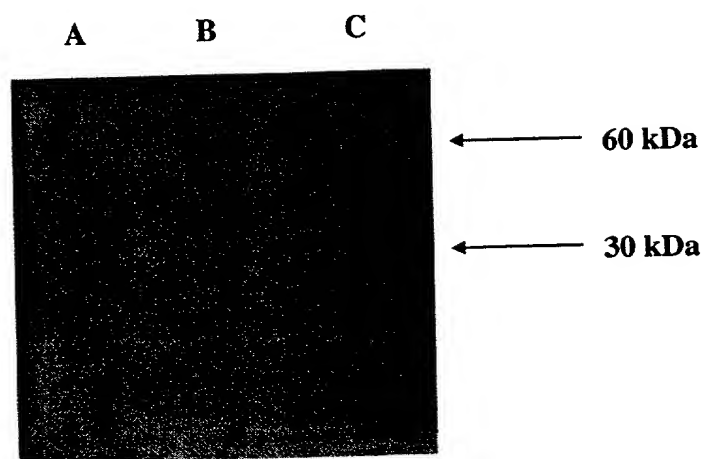
Activation of pAP-270 protein



- A. 100 ng of digested pAP-270
- B. 14.2 ng of digested pAP-270
- C. 2.0 ng of digested pAP-270
- D. 290 pg of digested pAP-270
- E. 46 ng of digested pAP-270
- F. Ricin A chain
- G. Negative control
- H. 46 pg of pAP-270
- I. 290 pg of pAP-270
- J. 2.0 ng of pAP-270
- K. 14.2 ng of pAP-270
- L. 100 ng of pAP-270

FIGURE 60

**Cleavage of pAP-288 protein by Plasminogen Tissue Activator (t-PA)**



**A. Ricin Standard (0.05 $\mu$ g)**

**B. pAP-288 (0.66  $\mu$ g) undigested**

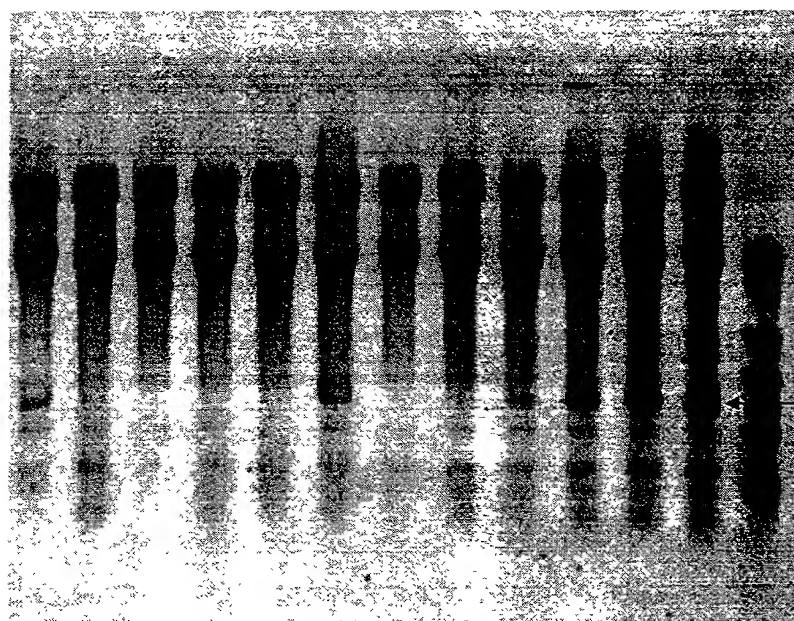
**C. pAP-288 (0.60  $\mu$ g) digested with 0.18  $\mu$ g of t-PA protease**



# FIGURE 61

## Activation of pAP-288 protein

A B C D E F G H I J K L M



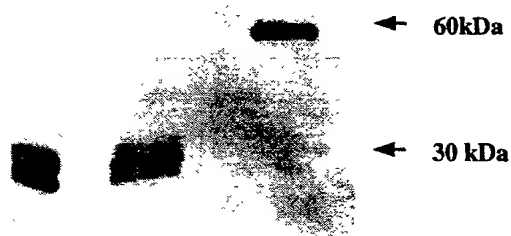
390 b  
fragment

- A. 200 ng of pAP-288
- B. 28.4 ng of pAP-288
- C. 4.0 ng of pAP-288
- D. 482 pg of pAP-288
- E. 83.4 pg of pAP-288
- F. Ricin A chain
- G. Negative control
- H. 83.4 pg of pAP-288 digested with tissue Plasminogen Activator (t-PA)
- I. 482 pg of pAP-288 digested with t-PA
- J. 4.0 ng of pAP-288 digested with t-PA
- K. 28.4 ng of pAP-288 digested with t-PA
- L. 200 ng of pAP-288 digested with t-PA
- M. RNA ladder

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FIGURE 62

**Cleavage of pAP 294 With Human Neutrophil Elastase**

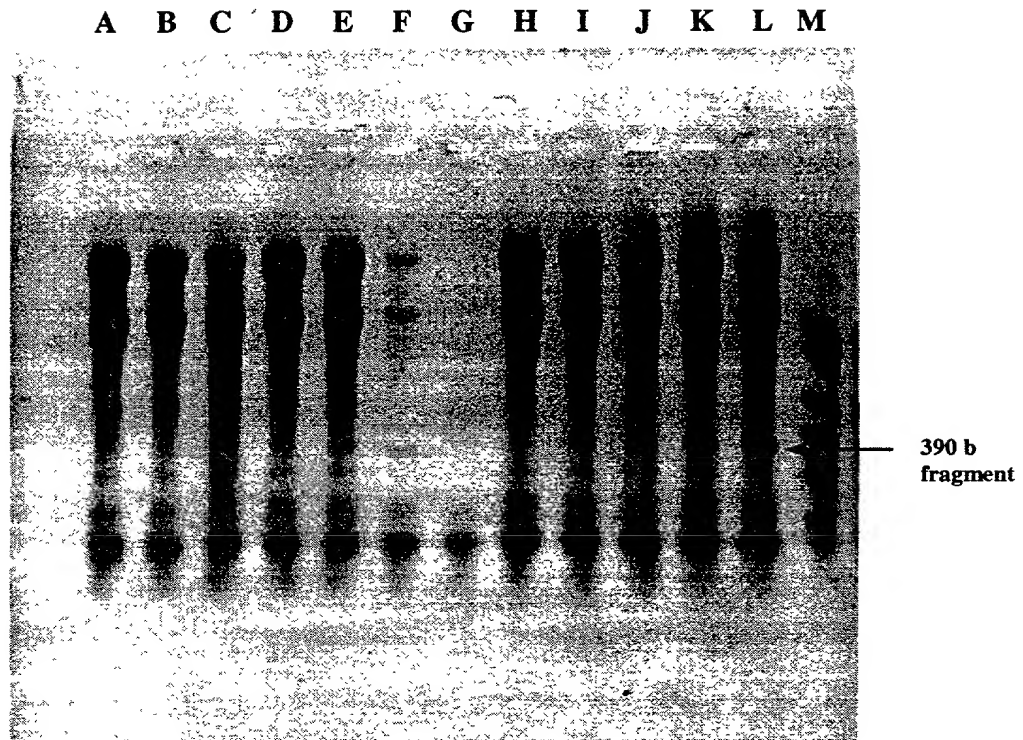


- A. Ricin Standard ( 0.050  $\mu$ g)
- B. pAP 294 protein ( 0.171  $\mu$ g) digested with 1.42  $\mu$ g of Human Neutrophil Elastase
- C. pAP 294 protein ( 0.121  $\mu$ g)

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**FIGURE 63**

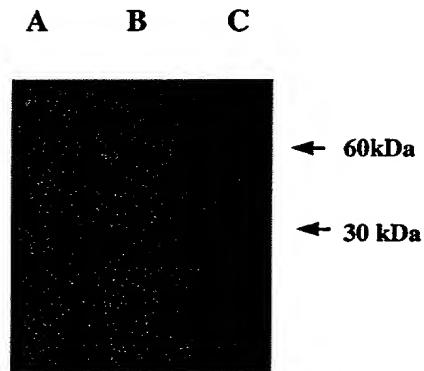
**Activation of pAP 294 Protein**



- A. 60 ng of pAP 294
- B. 8..57 ng of pAP 294
- C. 1.22 ng of pAP 294
- D. 175 pg of pAP 294
- E. 25 pg of pAP 294
- F. Ricin A chain
- G. Negative Control
- H. 360 ng of pAP 294 digested with Human Neutrophil Elastase
- I. 51 ng of pAP 294 digested with Human Neutrophil Elastase
- J. 7.3 ng of pAP 294 digested with Human Neutrophil Elastase
- K. 1.0 ng of pAP 294 digested with Human Neutrophil Elastase
- L. 150 pg of pAP 294 digested with Human Neutrophil Elastase
- M. RNA ladder

**FIGURE 64**

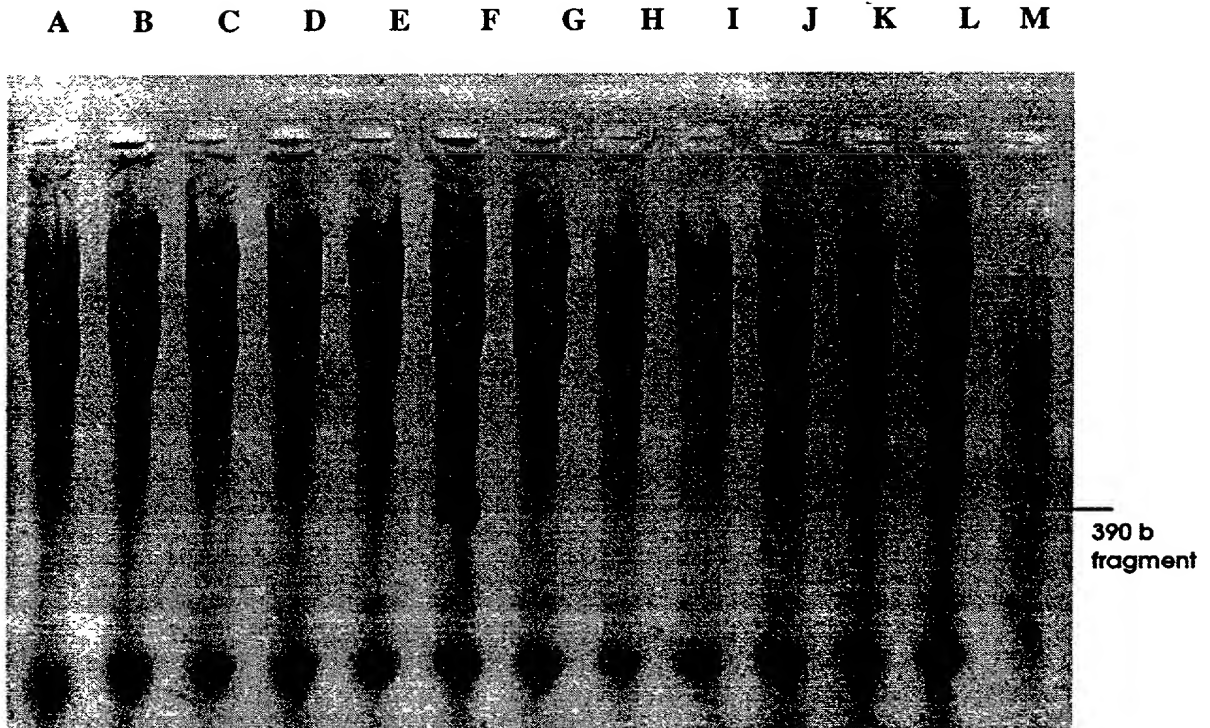
**Cleavage of pAP 296 with Calpain**



- A. Ricin Standard (0.05  $\mu$ g)  
B. pAP 296 (0.761  $\mu$ g) undigested  
C. pAP 296 (0.761  $\mu$ g ) digested with 4.0  $\mu$ g of Calpain

# **FIGURE 65**

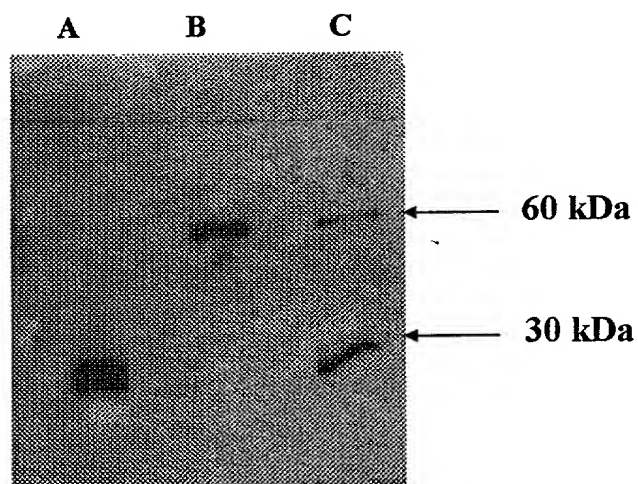
## **Activation of pAP 296 Protein**



- A. 100 ng of pAP 296 variant**
- B. 14.2 ng of pAP 296 variant**
- C. 2.0 ng of pAP 296 variant**
- D. 290 pg of pAP 296 variant**
- E. 46 pg of pAP 296 variant**
- F. Ricin A chain**
- G. Negative control**
- H. 46 pg of pAP 296 variant digested with Calpain**
- I. 290 pg of pAP 296 variant digested with Calpain**
- J. 2.0 ng of pAP 296 variant digested with Calpain**
- K. 14.2 ng of pAP 296 variant digested with Calpain**
- L. 100 ng of pAP 296 variant digested with Calpain**
- M. RNA ladder**

# **FIGURE 66**

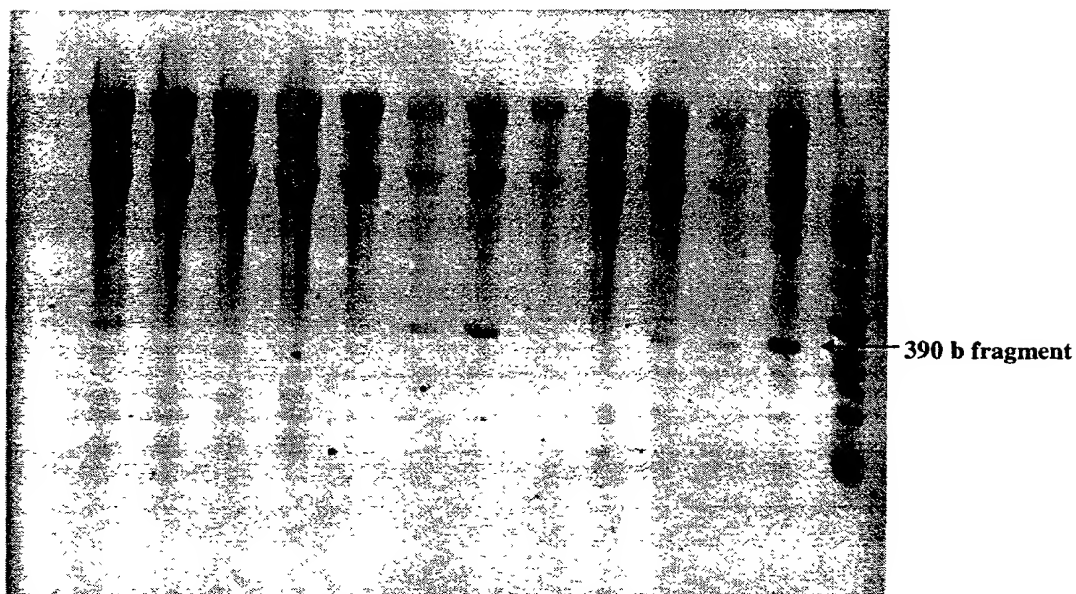
**Cleavage of pAP-222 Protein by The Matrix Metalloproteinase 2 (MMP-2)**



- A. Ricin Standard (0.250 ug)**
- B. pAP-222 Protein (0.250 ug)**
- C. pAP-222 protein ( 0.250 ug) digested with 0.28 ug of MMP-2**

**FIGURE 67****Activation of pAP-222 Protein**

A B C D E F G H I J K L M



- A. 100 ng of pAP-222 variant
- B. 14.2 ng of pAP-222 variant
- C. 2.0 ng of pAP-222 variant
- D. 291 pg of pAP-222 variant
- E. 41.7 pg of pAP-222 variant
- F. Ricin A chain
- G. Ricin A chain
- H. 41.7 pg of pAP-222 digested with MMP-2
- I. 291 pg of pAP-222 digested with MMP-2
- J. 2.0 ng of pAP-222 digested with MMP-2
- K. 14.2 ng of pAP-222 digested with MMP-2
- L. 100 ng of pAP-222 digested with MMP-2
- M. RNA ladder

## BERESKIN &amp; PARR

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Attorney Docket Number 10447-011

First Named Inventor Thor Borgford

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As a below named inventor, I hereby declare that

My residence, post office address, and citizenship are as stated below next to my name.

I believe I am the original first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

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I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment specifically referred to above.

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C. Lloyd Barghison	28,246	Robin L.A. Coster	26,016
Timothy J. Blinn	31,083	Michael E. Orlins	33,026

☒ Additional registered practitioner(s) names on supplemental Registered Practitioner Information sheet PTO/SB/02C attached hereto.

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Name of Sole or First Inventor:	<input type="checkbox"/> A petition has been filed for this unsigned inventor	
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THOR	BORGFORD	
Inventor's Signature	APPL 17 Date 2/2/00	
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City	Burnaby	Country
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☐ Additional inventors are being named on the supplemental Additional Inventor(s) sheet(s) PTO/SB/02A attached hereto

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Name	Registration Number	Name	Registration Number
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Andrew I. McIntosh	40,453	Stephen M. Beney	41,563
Shawn D. Jacka	43,379		

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Variable	Mean	SD	Min	Max
Age	31.1	4.2	22	40
Gender	0.5	0.5	0	1
Marital Status	0.3	0.5	0	1
Education	12.5	1.5	10	15
Income	1500	500	500	3000
Health Status	0.8	0.2	0	1
Stress Level	3.5	1.0	1	5
Life Satisfaction	4.2	0.8	3	5
Work-Life Balance	3.8	0.9	2	5
Family Support	4.5	0.7	3	5
Community Involvement	3.2	0.6	2	4
Personal Growth	4.0	0.8	3	5
Overall Well-being	4.1	0.7	3	5